REMARKS

I. Status of the Claims

Applicant's amendment of Feburary 1, 2005, amending claims 16 and 25 has been entered. Claims 1-15, 17, and 26 have been cancelled. Claims 16, 18-25 and 27-33 are pending and currently under consideration in the present application.

II. Written Description Rejection under 35 U.S.C. § 112, first paragraph

Claims 21-24 and 30-33 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. In particular, the Examiner contends that the specification does not set forth any written description supporting the limitation of claim 21 "(a) blocks the formation of a heterodimer containing the 40 kD subunit; or (b) allows the formation of a heterodimer containing the 40 kD subunit, but blocks the activity of said heterodimer" or the limitation of claim 30 "(a) blocks the formation of a heterodimer containing the 35 kD subunit; or (b) allows the formation of a heterodimer containing the 35 kD subunit, but blocks the activity of said heterodimer." Office Action of 05/17/05, p. 2.

In responding to Applicants' remarks of February 1, 2005, the Examiner continues to assert that "it is not clear how IL-12 can be used to treat autoimmune conditions," and to ask "[h]ow is IL-12 or a IL-12 subunit that retains IL-12 biological activity used to treat an autoimmune condition, when the art teaches that IL-12 causes autoimmune conditions?" Office Action of 05/17/05, p. 4. The currently rejected claims depend from independent claim 16, which is directed to "at least one antagonist that binds with a 40 kD subunit of IL-12" (emphasis added). Applicants respectfully note that the claimed invention as amended herein does not use IL-12 or an IL-12 subunit to treat

autoimmune conditions, but only **antagonists** directed to heterodimeric IL-12 or to the 40 kD IL-12 subunit.

A. The claimed limitations are implicit in the disclosure of the specification as-filed

The Examiner first alleges that the specification does not provide implicit disclosure for the claimed limitations because, although it teaches that IL-12 antagonists include "species that will bind IL-12 **or biologically active fragments thereof**," neither IL-12 subunit displays any biological function alone. Specification p. 6, lines 21-22; Office Action of 05/17/05, p. 4. The Examiner cites an article from the primary literature in support of this proposition. *See* Kim *et al.* "The role of IL-12 in inflammatory activity of patients with rheumatoid arthritis (RA)," *Clin. Exp. Immunol.* 119:175-181 (2000) ("Kim"). Kim teaches that IL-12 is a disulfide-linked heterodimer comprising a p35 and a p40 subunit, "neither of which has been found to display any significant biological function alone." Kim, p. 175.

Applicants respectfully assert that, although Kim discusses that the individual IL-12 subunits alone do not have the same biological activity as the IL-12 heterodimer, this does not mean that they lack all biological activity. Indeed, both subunits display biological activity in binding together to form heterodimeric IL-12, a protein with a different biological activity. The statement cited in Kim is directed to whether the subunits alone have any IL-12 activity. Reducing or eliminating the ability of either IL-12 subunit to assemble into functional IL-12 by administering an antibody to that subunit will ultimately reduce the biological activity of IL-12, because functional IL-12 requires association of both subunits together. Sequestration of one subunit directly affects the

amount of IL-12 formed. Therefore, the specification implicitly supports the use of antagonists of either the p35 or the p40 subunit because the phrase "**biologically active fragments thereof**" in the specification encompasses the individual subunits as well as other fragments. Specification p. 6, lines 21-22.

The specification teaches that IL-12 is a heterodimeric protein comprising one 35 kD and one 40 kD subunit, and provides methods of producing each subunit recombinantly. Specification p. 8, lines 7-8; p. 9, lines 11-18. The specification also provides suitable expression vectors and host cells that may be used for expressing IL-12 or the p35 and p40 subunits thereof. Specification, p. 10, lines 1-14. In addition, the specification discusses biochemical purification techniques that can be used for purifying IL-12 or a subunit thereof. Specification p. 10, line 15 to p. 12, line 13. Finally, the specification teaches that IL-12 or an IL-12 subunit can be used to make an IL-12 antagonist. Specification p. 7, line 12 to p. 8, line 4. Accordingly, based on the specification, one of ordinary skill in the art could easily purify IL-12, or its individual subunits, for use in obtaining an IL-12 antagonist.

The specification provides antibodies as an example of such antagonists, including monoclonal antibodies, polyclonal antibodies, chimeric antibodies and fragments thereof. Specification p. 7, lines 1-5. The specification also teaches that "[p]olyclonal sera and antibodies to IL-12 can be produced by inoculation of a mammalian subject with IL-12 or **fragments thereof** in accordance with known methods." Specification p. 7, lines 17-19. Applicants note that disclosure of a fully characterized antigen, whether "by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository" provides adequate written

description of an antibody claimed by its binding affinity to that antigen. *Noelle v. Lederman*, 355 F.3d 1343, 1349 (Fed. Cir. 2004); MPEP § 2163 II.A.3.a (8th ed. Rev. 2, May, 2004).

Accordingly, it would be clear to one of ordinary skill in the art that antibodies that bind IL-12 or biologically active fragments thereof may be directed to heterodimeric IL-12, to a particular subunit of IL-12, or to biologically active fragments thereof. Furthermore, the specification teaches that antagonists which block IL-12 function would be desirable, since such antagonists would prevent an increase in IFN-γ and/or TNF-α levels. Specification, p. 7, lines 8-10. Since IL-12 typically exists as a disulfide-linked heterodimer, it would be clear to one of ordinary skill in the art that blocking IL-12 function could be accomplished either by blocking the formation of the active heterodimer or by blocking its activity after association of the subunits.

B. *In haec verba* support is not required to satisfy the written description requirement

Applicants respectfully assert that satisfying the written description requirement of 35 U.S.C. § 112, first paragraph, does not require *in haec verba* support for the claimed subject matter at issue. *See, e.g., Lampi Corp. v. American Power Products, Inc.*, 228 F.3d 1365, 1378 ("in order to satisfy the written description requirement, the disclosure as originally filed need not provide *in haec verba* support for the claimed subject matter at issue") (citing *Fujikawa v. Wattanasin,* 93 F.3d 1559, 1570 (Fed. Cir. 1996)). Instead, the specification "need only reasonably convey to persons skilled in the art that the inventor had possession of the subject matter in question." *Fujikawa,* 93 F.2d at 1570.

Applicants respectfully submit that the specification as-filed provides adequate support for the claim limitations at issue. Applicants respectfully assert that one of ordinary skill in the art would have recognized, based on the specification as-filed, that Applicants' invention encompassed both antagonists which block formation of the active IL-12 heterodimer containing both p35 and p40 subunits, and antagonists which allow the formation of the heterodimer but block its activity.

In view of the foregoing remarks, Applicants respectfully submit that the instant specification provides adequate written description support for antibodies which bind the p40 subunit of IL-12, including antibodies which either block the formation of the heterodimer containing both p35 and p40 subunits, or allow the formation of the heterodimer but block its activity. Therefore Applicants respectfully request that this rejection be withdrawn.

III. Enablement Rejection under 35 U.S.C. § 112, first paragraph

Claims 16, 18-25 and 27-33 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. In particular, the Examiner alleges that the specification does not teach an antagonist or an antibody that binds to the 40 kD or the 35 kD subunit of IL-12 "that can be used to treat any autoimmune condition . . . in a human," and concludes that the specification at pages 6-8 appears to be "a mere paper protocol for a method of treating autoimmune conditions." Office Action of 05/17/05, p. 6. The Examiner further alleges that "the specification has not set forth enablement for the scope of treating autoimmune conditions as defined" in the specification, because "there are no known compositions that can be administered to a subject that will prevent an autoimmune condition or

disease." Office Action of 05/17/05, p. 7. Finally, the Examiner alleges that "the state of the art has not shown definitively that IL-12 antagonists or antagonists (i.e. antibodies) that bind the 40 kD subunit of IL-12 or the 35 kD subunit of IL-12 can be used to treat or prevent the numerous autoimmune conditions or diseases" as claimed. *Id.* at pp. 7-8.

Applicants respectfully traverse this rejection. Applicants respectfully assert that the instant specification enables a person of ordinary skill in the art to make and use antagonists that bind with a 40 kD subunit of IL-12 in methods of treating autoimmune conditions promoted by an increase in IFN-γ and/or TNF-α levels in a human subject.

A. The specification enables treatment of autoimmune conditions promoted by an increase in IFN-γ and/or TNF-α levels in a human subject

The Examiner alleges that the specification does not teach an antagonist or an antibody that binds to the 40 kD or the 35 kD subunit of IL-12 "that can be used to treat any autoimmune condition . . . in a human," and concludes that the specification at pages 6-8 appears to be "a mere paper protocol for a method of treating autoimmune conditions." Office Action of 05/17/05, p. 6.

Applicants respectfully observe that the claimed invention does not encompass methods of treating "any autoimmune condition . . . in a human," but only those promoted by an increase in IFN-γ and/or TNF-α levels in a human subject. Those disorders include, but are not limited to, multiple sclerosis ("MS"), insulin-dependent diabetes melitis ("IDDM"), rheumatoid arthritis ("RA"), systemic lupus erythematosus ("SLE"), autoimmune thyroiditis, autoimmune uveoretinitis, and Guillain-Barre syndrome. Specification p.1, line 7 to p. 2, line 2.

The Examiner appears to be arguing that experiments showing that a polyclonal antibody targeting IL-12 effectively treated EAE in mice do not support a similar use to treat MS in humans. Applicants respectfully assert that data generated from testing in an animal model "almost invariably will be sufficient" to establish therapeutic or pharmacological utility for a compound, composition or process. MPEP § 2107.03 III. Such data need not be obtained from an art-recognized animal model for the particular disease or condition to which the asserted utility relates, provided the data presented "reasonably correlates" to the asserted utility. *Id.* Applicants need only supply "a reasonable evidentiary showing" in support of an asserted therapeutic utility—"absolute certainty is not required by the law." *In re Wooddy & Moore,* 331 F.2d 639 (C.C.P.A. 1964); MPEP § 2107.03 III.

IL-12 levels correlate well with disease activity, and administration of recombinant IL-12 to mice after induction of EAE accelerates progression to peak disease and prolongs duration of symptoms. Specification p. 19, line 3 to p. 20, line 5. The specification teaches the treatment of experimental autoimmune encephalomyelitis ("EAE") with a sheep polyclonal antibody directed to murine IL-12. Specification p. 20, line 8 to p. 21, line 1. The antibody was raised against mouse IL-12 which, like human IL-12, is a heterodimer comprising disulfide-linked p35 and p40 subunits. The specification also teaches that EAE "is widely recognized as an acceptable animal model for multiple sclerosis in primates." Specification, p. 14, lines 21-22.

EAE was induced in mice following the adoptive transfer of lymphocytes from mice immunized with myelin proteolipid protein (PLP) and restimulated in vitro with a synthetic PLP peptide. Specification, p. 15, lines 2-4. Administration of the polyclonal

IL-12 antibody during the first six days following the induction of EAE delayed the onset of clinical signs by approximately seven days compared to control animals treated with an equal amount of sheep immunoglobulin. Specification p. 20, lines 11-16. Administration of the antibody every other day for twelve days after induction of EAE delayed disease onset even further, and dramatically reduced clinical signs of disease. Specification p. 20, line 18 to p. 21, line 1. These data suggest that treatment of EAE or multiple sclerosis with IL-12 antagonists may delay or even prevent onset of symptoms.

Applicants respectfully note that numerous, well-accepted animal models have been developed for the study of other human autoimmune disorders, including:

(1) non-obese diabetic ("NOD") mice, an accepted animal model for IDDM, Campbell et al., "Essential Role for Interferon-γ and Interleukin-6 in Autoimmune Insulin-Dependent Diabetes in NOD/Wehi Mice," *J. Clin. Invest.* 87:739-742 (1991);

(2) collagen-induced arthritis in mice, an accepted model for rheumatoid arthritis, Knoerzer et al., "Collagen-Induced Arthritis in the BB Rat: Prevention of Disease by Treatment with CTLA-4-Ig," *J. Clin. Invest.* 96:987-993 (1995);

(3) experimental autoimmune thyroiditis ("EAT") in mice, an accepted model for Hashimoto's thyroiditis, Tang et al., "The Effects of a Monoclonal Antibody to Interferon-γ on Experimental Autoimmune Thyroiditis (EAT): Prevention of Disease and Decrease of EAT-specific T Cells," *Eur. J. Immunol.* 23:275-278 (1993);

(4) BXSB mice, an accepted model for systemic lupus erythematosus ("SLE"), Merino et al., "Prevention of Systemic Lupus Erythematosus in Autoimmune BXSB mice by a Transgene Encoding I-E α Chain," *J. Exp. Med.* 178:1189-1197 (1993); and

(5) experimental autoimmune uveoretinitis ("EAU") in rats and mice, an accepted animal model of autoimmune intraocular inflammation, Charteris et al., "Interferon-gamma (IFN-γ) Production In Vivo in Experimental Autoimmune Uveoretinitis," *Immunolog.* 75:463-467 (1992).

Applicants respectfully contend that well-established animal models of human disease are routinely used to develop candidate pharmaceutical compositions and methods of treatment later used in humans. Testing to establish the suitability of such compositions and methods for human use is within the level of ordinary skill in the art and does not require undue experimentation.

In view of the foregoing remarks and amendments, Applicants respectfully assert that the instant specification enables a person of ordinary skill in the art to make and use antagonists that bind with the 35 kD or 40 kD subunit of IL-12 in methods of treating autoimmune conditions promoted by an increase in IFN-γ and/or TNF-α levels in a human subject. Therefore Applicants respectfully request that this rejection be withdrawn.

B. The specification enables the treatment of autoimmune conditions as defined in the specification

The Office further alleges that the disclosure does not enable "the scope of treating autoimmune conditions as defined" in the specification, because "there are no known compositions that can be administered to a subject that will prevent an autoimmune condition or disease," but offers no evidence in support of this conclusory assertion. Office Action of 05/17/05, p. 7. Applicants respectfully disagree with the Office's contention.

On the contrary, treatment with immunosuppressive compounds has been shown to prevent onset of IDDM in animal models. For example, administration of rapamycin prevented the onset of IDDM in NOD mice compared to control animals, and administration of FK506 prevented the onset of autoimmune insulitis and diabetes in NOD mice compared to control animals. Baeder et al., "Rapamycin prevents the onset of insulin-dependent diabetes mellitus (IDDM) in NOD mice," *Clin. Exp. Immunol.* 89(2):174-178 (1992); Kai et al., "Prevention of insulitis and diabetes in nonobese diabetic mice by administration of FK506," *Transplantation* 55(4):936-940 (1993). In addition, treatment of BXSB mice with a transgene encoding a class II major histocompatibility complex antigen, I-E α, prevented the mice from developing SLE. Merino et al., *supra*. Finally, administration of a CTLA-4-immunoglobulin fusion protein targeting cell surface molecules involved in T cell costimulation prevented development of clinical manifestations of collagen-induced arthritis in BB/Wor mice. Knoerzer et al., *supra*. Applicants provide copies of these references for the Examiner's consideration as a courtesy.

In view of the foregoing remarks and amendments, Applicants respectfully assert that the instant specification enables "the scope of treating autoimmune conditions as defined" in the specification. Therefore Applicants respectfully request that this rejection be withdrawn.

C. The specification enables one of ordinary skill in the art to make and use the invention without undue experimentation

The Examiner alleges that the art is unpredictable, because (1) "it is difficult to predict which antibody to the subunits of these cytokines (IL-12 or IL-23) or epitopes of the subunits of these cytokines binding to IL-12 are effective in a method of treating autoimmune diseases, without experimental evidence" and (2) "the state of the art has not shown definitively that IL-12 antagonists or antagonists (i.e. antibodies) that bind the 40 kD subunit of IL-12 or the 35 kD subunit of IL-12 can be used to treat or prevent the numerous autoimmune conditions or diseases" as claimed. Office Action of 05/17/05, at pp. 6-8. As the Examiner notes, "[t]he state of the art . . . teaches that . . . IL-12, a heterodimer of p40 and p35 that are disulfate-bonded causes increases in IFN-γ and/or TNF-α levels and that this increase causes an autoimmune disease or condition." Office Action of 05/17/05, p. 8. Because the art teaches that active IL-12 must be in the form of a heterodimer containing both p40 and p35, one of ordinary skill in the art would recognize that an antibody specific for a subunit of IL-12 would effectively antagonize that cytokine because it would deplete levels of that subunit, reducing the total amount of that subunit available to form active heterodimer.

The Examiner again appears to be arguing that Applicants have not "definitively" proven that the claimed methods and IL-12 antagonists will be therapeutically effective,

because the cytokines involved in the etiology and progression of various autoimmune disorders are not known to a certainty. Applicants respectfully remind the Examiner that "absolute certainty is not required by the law." *In re Wooddy & Moore,* 331 F.2d 639, 141 USPQ 518, 520 (C.C.P.A. 1964); MPEP § 2107.03 III. Applicants respectfully submit that the experimental data disclosed in Example 1 establishes the required "reasonable correlation between the activity in question and the asserted utility," and therefore enables one of ordinary skill in the art to make and use the claimed invention. MPEP § 2107.03 I. The disclosure of the references cited by the Examiner only provides additional support for the claimed invention.

The Examiner cites Benson et al., Fox et al., and Becher et al. in support of the contention that "it is difficult to predict which antibody to the subunits of these cytokines (IL-12 or IL-23) or epitopes of the subunits of these cytokines binding to IL-12 are effective in a method of treating autoimmune diseases, without experimental evidence." *Id.* at p. 6; Benson et al., "The Role of IL-23 in Experimental Autoimmune Encephalomyelitis," *FASEB J.* 16(5):A1045 (2002) ("Benson"); Fox et al., "Anti-Interleukin-12 Antibody: Potential Role in Preventing Relapses of Multiple Sclerosis," *BioDrugs* 13(4):233-241 (2000); and Becher et al., "Experimental Autoimmune Encephalitis and Inflammation in the absence of Interleukin-12," *J. Clin. Invest.* 110(4):493-497 (2002). Applicants respectfully assert that, on the contrary, these references instead suggest that the IL-12 heterodimer is an effective target for antibody therapy, and thereby provide further support for the enabling disclosure of the claimed invention.

Benson compared EAE disease progression in mice treated with a neutralizing monoclonal antibody specific for IL-12 p35 or treated with a neutralizing monoclonal antibody specific for IL-12 p40, which targeted both IL-12 and IL-23, because p40 is present in both cytokines. Results based on clinical score analysis, histopathology, *in vitro* proliferation assays, and cytokine expression profiles indicated that, while neutralization of IL-12 p35 had no effect on EAE progression, neutralization of p40—present in both IL-12 and IL-23—"effectively ameliorated EAE clinical signs." Benson et al. *supra*, at A1045. The Examiner alleges that, based on these results, "it is difficult to predict which antibody to the subunits of these cytokines (IL-12 or IL-23) . . . [would be] effective in a method of treating autoimmune diseases." On the contrary, Applicants note that Benson unambiguously demonstrates that a neutralizing antibody directed to the p40 subunit of IL-12 can successfully treat EAE in mice.

Fox, published after this application was filed, reviewed the role of IL-12 in MS, discussed the possible use of anti-IL-12 antibody in treating MS and other autoimmune disorders, and noted that "[a]ntibodies against immune mediators are used in several disorders to modulate the immune response." Fox et al. *supra*, at 237. Fox also observed that monoclonal antibodies have been produced that block the biological activity of IL-12 by blocking its binding to the IL-12 receptor. *Id.* Fox further discussed studies showing that administration of a monoclonal antibody specific for IL-12 to mice after injection with encephalitogenic lymphocytes protected them from developing EAE. *Id.* Finally, Fox observed that "[a]nimal models for the other autoimmune disorders mentioned above are also sensitive to anti-IL-12 antibodies." *Id.* at 238. Such antibodies have been shown (1) to block the development of EAU in mice, (2) to inhibit

or ameliorate the progression of Crohn's disease, and (3) to delay the onset and attenuate the clinical and pathological severity of collagen-induced arthritis in mice. *Id.* Thus Fox clearly demonstrates that an antibody directed to active IL-12 heterodimer—which must contain the p40 subunit—can successfully treat a number of autoimmune disorders.

Becher, also published after this application was filed, examined the role of IL-12 subunits individually in the progression of EAE in mice. Becher et al., *supra*, at 493. Using transgenic mice lacking either p35 or p40, Becher demonstrated that mice lacking p35 remain susceptible to EAE, while mice lacking p40 are resistant to EAE, and concluded that, although the IL-12 heterodimer is not essential for the induction and progression of EAE, its p40 subunit is "absolutely critical" for the development of EAE. *Id.* Thus Becher clearly suggests that an antagonist directed to the p40 subunit of IL-12 can successfully inhibit development of EAE in mice.

Finally, the Examiner cites Adorini in support of the proposition that "the state of the art has not shown definitively that IL-12 antagonists or antagonists (i.e. antibodies) that bind the 40 kD subunit of IL-12 or the 35 kD subunit of IL-12 can be used to treat or prevent the numerous autoimmune conditions or diseases as claimed." Adorini, "Immunotherapeutic approaches in multiple sclerosis," *J. Neurol. Sci.* 223:13-24 (2004). Adorini reports that, because IL-12-dependent T_H1 responses have been implicated in a number of autoimmune disorders, including IDDM, EAE, collagen-induced arthritis, EAU, granulomatous colitis, experimental autoimmune myasthenia gravis, and thyroiditis, "targeting IL-12 may prove beneficial in some forms of MS . . . and it is likely that IL-12 antagonists can be useful in other autoimmune conditions." *Id.* at 17. Thus,

like all the art cited by the Examiner, Adorini suggests that "it is likely" IL-12 antagonists can be useful in treating other autoimmune conditions.

In view of the foregoing remarks and amendments, Applicants respectfully assert that the instant specification enables one of ordinary skill in the art to make and use the claimed invention without undue experimentation. Therefore Applicants respectfully request that this rejection be withdrawn.

IV. Nonstatutory Double Patenting Rejection

Claims 16, 18-22 and 27-31 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 16 and 22 of co-pending U.S. Patent Application No. 09/512,701, issued on December 14, 2004, as U.S. Patent No. 6,830,751.

Applicants respectfully traverse this rejection and ask that it be held in abeyance until allowable subject matter is determined. At that time, Applicants will consider whether to file a Terminal Disclaimer.

CONCLUSION

In view of the foregoing remarks and amendments, Applicants respectfully request the entry of this Amendment, the Examiner's reconsideration and the timely allowance of all pending claims as amended. Should the Examiner feel that this application is not in condition for allowance, Applicants request that the Examiner contact the undersigned representative at 202-408-4086.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

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Rapamycin prevents the onset of insulin-dependent diabetes mellitus (IDDM) in NOD mice

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(Accepted for publication 30 April 1992)

SUMMARY

The effect of the immunosuppressive agent rapamycin (RAPA) was assessed in the non-obese diabetic (NOD) mouse which is an autoimmune model of IDDM. RAPA was prepared in a vehicle of 8% cremophor EL/2% ethanol and investigated in two studies. NOD/MrK female mice (six per group, study no. 1; 10 per group, study no. 2) were dosed three times per week p.o. by gavage from 56 to 170 days of age (study no. 1) or from 64 to 176 days of age (study no. 2). Mice treated with RAPA at 0·6 mg/kg, 6 mg/kg, or 12 mg/kg maintained normal plasma glucose through 170 or 176 days of age with 10%, 0%, and 0% incidence of diabetes respectively. In contrast, naive, vehicle-treated, or RAPA 0·06 mg/kg-treated mice exhibited elevated plasma glucose and disease incidence typical for female NOD mice. Mice which became diabetic had elevated levels of β-hydroxybutyrate, triglycerides and cholesterol. These plasma lipid concentrations were positively correlated with the duration of hyperglycaemia ($r = 0{\cdot}85$, 0·87 and 0·84 respectively). Outside of its ability to prevent diabetes, RAPA itself did not affect the lipid profile of the mice. Intervention therapy with RAPA was ineffective at reversing the course of disease after IDDM onset under these experimental conditions. Finally, we report here that prophylactic treatment with RAPA was able to protect against IDDM development in some RAPA-treated mice 41 weeks after cessation of treatment. These data show that orally administered RAPA is effective in preventing onset of disease in the NOD mouse, a relevant model of autoimmune type I diabetes in man.

Keywords autoimmunity insulin-dependent diabetes mellitus NOD mice rapamycin

INTRODUCTION

The non-obese diabetic mouse (NOD), an autoimmune model of type I IDDM, exhibits approximately 70% incidence of diabetes at 160 days of age [1,2]. Genetic susceptibility involving class II MHC genes plays a major role in this autoimmune disease for both humans and NOD mice [3–8]. The pancreatic islets become infiltrated with lymphocytes (insulitis) and insulin-producing beta cells are destroyed. Adoptive transfer studies in NOD mice [9] have shown that T cell-mediated events occur initially in IDDM while humoral abnormalities (cytoplasmic islet cell, insulin, and 64-kD protein autoantibodies) contribute later during the disease progression (see review [10]).

Rapamycin (RAPA) is a novel macrolide which appears to have a different mechanism of action *in vitro* from the immunosuppressants cyclosporin A (CsA) and the macrolide FK506 [11] (see review [12–14]). Rapamycin appears to affect both calcium-dependent and independent pathways of T cell proliferation by working later in the cell cycle (G_1 phase) as compared with FK506 or CsA which primarily act on calcium-dependent pathways in the earlier G_0 phase. Rapamycin, unlike FK506 or CsA, has little effect on mRNA levels for IL-2, IL-2 production, or IL-2R expression, but does have the ability to inhibit proliferation stimulated by exogenous addition of this cytokine as well as IL-4.

Rapamycin is efficacious in animal models of transplantation in suppressing rejection and prolonging survival time of both skin [15] and organ allografts (see review [13]). Rapamycin also demonstrates an immunosuppressive effect in murine T cell-mediated autoimmune models including collagen-induced arthritis (CIA) [16] and the MRL lupus model [17]. Since CsA and FK506 have been reported to prevent onset of IDDM in both the NOD mouse [1,2,18] and BB rat models [19,20], and CsA has partial clinical efficacy in patients with recent IDDM onset [21], we studied the effects of rapamycin in the NOD model and analysed incidence of diabetes by measuring plasma glucose, water consumption, and body weight at regular

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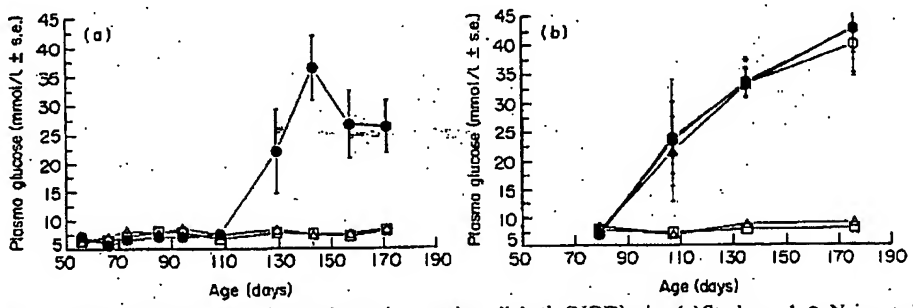

Fig. 1. The effect of rapamycin (RAPA) on plasma glucose in non-obese diabetic (NOD) mice. (a) Study no. 1. ●, Naive; △, RAPA 6 mg/kg; □, RAPA 12 mg/kg. (b) Study no. 2. ●, Naive; ▲, vehicle; ○, RAPA 0·06 mg/kg; □, RAPA 0·6 mg/kg; △, RAPA 6 mg/kg. *Elevated plasma glucose observed in one of 10 mice from the RAPA 0·6 mg/kg group.

intervals. In addition, a cross-sectional evaluation of plasma lipids including triglyceride, cholesterol, and β-hydroxybutyrate was conducted.

MATERIALS AND METHODS

Two studies (nos 1 and 2) were used to determine the effects of rapamycin in the NOD model.

Treatment protocol

Female NOD/MrKTACfBR mice (Taconic), 8 weeks of age (study no. 1) or 9 weeks of age (study no. 2), were housed 3–4 mice per cage in a barrier facility and given food and water *ad libitum*. Weight and water consumption were measured on a weekly basis. In the initial study, six mice per group were randomly distributed into three groups including naive control, RAPA 6 mg/kg, and RAPA 12 mg/kg. Rapamycin was dissolved in a vehicle containing 8% cremophor EL/2% ethanol and administered in a volume of 0·1 ml/10 g body weight. In the second study, 10 mice per group were randomly distributed into six groups (A–F) as described below. The effects of vehicle (A) as compared to naive control (B) were tested. A dose response to rapamycin (0·06, 0·6 and 6 mg/kg (C–E)) was determined and the ability to reverse diabetes with RAPA 6 mg/kg immediately after onset (F) was assessed. Administration of drug began at 56 days (study no. 1) and 64 days (study no. 2) and continued three times per week p.o. by gavage until mice were killed at 170 days of age (study no. 1 only) and through 176 days (study no. 2). Non-diabetic rapamycin-treated mice from study no. 2 were then followed for an additional 41-week period after cessation of rapamycin and analysed for incidence of IDDM.

Biochemical analysis

At regular intervals blood was collected from the tail vein into tubes containing sodium fluoride and plasma removed for analysis of glucose using the Abbott Biochromatic Analyzer. A plasma glucose level consistently > 11·1 mmol/*l* was the criterion used to determine overt onset of diabetes. On day 176 of study no. 2, blood was collected from the retroorbital sinus and plasma levels of triglyceride and cholesterol were investigated using the above method. Plasma levels of β-hydroxybutyrate were analysed enzymatically using the method of Williamson *et al.* [22].

Water consumption determination

Mice were housed 3–4 in cages, and thus water consumption (millilitres of water consumed/mouse per day) was calculated by measuring total consumption (ml) for a 7-day period in a cage and dividing by the total number of mice housed. This was done for each of the 2–3 cages per treatment group. A final weighted mean per group was determined by using consumption data per mouse in a cage as a value for each of the mice housed in that cage and then averaging these values for each of the mice in the group. Although this method does lead to considerable variation by accounting for water consumption of both diabetic and non-diabetic mice, and does not allow for calculation of statistical differences between groups, it did serve as a valuable non-invasive indicator of new onset and as an indicator of osmotic diuresis due to glycouria.

Statistical analysis

The data were expressed as mean ± s.e. Fisher's exact test was used for comparison of per cent incidence of diabetes between the groups. Student's *t*-test was used for comparison of weight between groups and two-way analysis of variance was used to determine significance between treatment groups for the plasma lipid levels. All significance levels were set at $P < 0{\cdot}05$. Finally, Pearson's correlation, set at the 95% confidence interval, was used to compare lipid levels and the number of days of overt diabetes.

RESULTS

Incidence of diabetes

In the initial study, rapamycin-treated mice at 170 days of age had no incidence of diabetes (0/10) which was significantly lower from control incidence (4/6, 67%) at $P = 0{\cdot}008$ by Fisher's exact test. In the second study, six out of ten (60%) vehicle-treated mice became diabetic by 176 days of age which was not significantly different from naive control mice (6/10, 60%). Rapamycin treatment again protected against diabetes at 6 mg/kg (0/10) and also at 0·6 mg/kg (1/10, 10%) which was significantly lower than incidence for naive or vehicle control at $P = 0{\cdot}005$ and $P = 0{\cdot}029$, respectively. A no effect concentration of rapamycin was determined at 0·06 mg/kg (60% incidence).

Plasma glucose levels

Plasma glucose levels for all groups were below 7·2 mmol/*l* (study no. 1) and below 8·3 mmol/*l* (study no. 2) at the beginning of the studies as shown in Figs 1a and b, respectively. Elevated plasma glucose levels for diabetic mice were first observed by 129 days of age in study no. 1 for naive control and by 107 days for naive control, vehicle, and RAPA 0·06 mg/kg in study no. 2.

Table 1. Plasma levels of β-hydroxybutyrate, triglyceride and cholesterol in non-obese diabetic (NOD) mice at 176 days of age

Treatment	β-hydroxybutyrate (mmol/l)		Triglyceride (mmol/l)		Cholesterol (mmol/l)	
	Non-diabetic	Diabetic	Non-diabetic	Diabetic	Non-diabetic	Diabetic
Naive	0·61±0·38 (4)	15·2±5·6 (4)*	1·24±0·22	5·06±1·14*	2·55±0·25	5·85±1·30*
Vehicle†	0·66±0·13 (4)	12·8±5·4 (4)*	0·92±0·06	4·06±1·00*	2·60±0·10	4·75±0·85*
RAPA 0·06 mg/kg†	0·45±0·03 (4)	7·6±5·4 (2)*	0·84±0·08	5·04±2·76*	2·65±0·05	4·85±0·50*
RAPA 0·6 mg/kg†	0·58±0·12 (9)		0·74±0·04		2·50±0·05	
RAPA 6 mg/kg†	0·52±0·08 (10)		1·30±0·06		2·90±0·10	

* $P < 0·05$ when compared to vehicle non-diabetic.
† Administered at 64 days of age and continued three times per week p.o.
Values are mean±s.e.m.
Numbers in parentheses are *n* values.

Table 2. Profile of rapamycin-protected mice after cessation of treatment

Weeks after cessation	RAPA 6 mg/kg, glucose (mmol/l)					RAPA 0·6 mg/kg, glucose (mmol/l)				
	Incidence (%)	Non-diabetic	Diabetic	Water	Status	Incidence (%)	Non-diabetic	Diabetic	Water	Status
1	0/10 (0)	—	—	5·2±0		0/9 (0)	—	—	4·7±0·2	
3	0/10 (0)	7·8±0·3 (10)	—	4·8±0		0/9 (0)	6·8±0·3 (9)	—	4·4±0·2	
6	0/10 (0)	6·8±0·3 (10)	—	5·1±0·1		1/9 (11)	6·8±0·2 (8)	13·9±0 (1)	4·7±0·2	
9	0/10 (0)	7·4±0·2 (10)	—	—		1/9 (11)	7·2±0·4 (8)	29·4±0 (1)	—	
14		—	—	4·0±0·1	†		—	—	9·4±2·6	
15	0/10 (0)	6·8±0·4 (9)	—	4·3±0·1	†	1/9 (11)	7·4±0·3 (8)	41·1±0 (1)	8·9±2·3	
20		—	—	5·1±0·2	†		—	—	5·3±0·4	‡
21	0/10 (0)	7·6±0·4 (7)	—	5·0±0·2		2/9 (22)	7·3±0·5 (7)	17·3±0 (1)	4·7±0·2	
24		—	—	5·4±0	†		—	—	5·5±0·3	
26	0/10 (0)*	10·1±1·6 (7)*	—	5·3±0·4		2/9 (22)	9·9±0·8 (7)	36·6±0 (1)	7·9±1·5	
29		—	—	5·5±0·3			—	—	10·8±2·5	‡
31		—	—	5·3±0·4			—	—	5·3±0·6	
33	0/10 (0)*	—	—	5·7±0·5		3/9 (33)	—	—	8·0±1·8	
35		—	—	4·6±0·3	†		—	—	4·3±0·2	‡
37	0/10 (0)*	8·0±1·3 (5)*	—	4·7±0·3		3/9 (33)	8·5±0·5 (6)	—	4·2±0·2	
39		—	—	4·5±0·6	†		—	—	4·3±0·2	
41	0/10 (0)*	9·7±1·8 (4)*	—	4·6±0·7		3/9 (33)	8·6±0·5 (6)	—	4·2±0·2	

* One mouse had a slightly elevated plasma glucose level from 361 to 466 days of age with no other clinical signs of IDDM including loss of weight or increased water consumption.
† Death not due to hyperglycaemia.
‡ Death as a result of diabetes.
Values are mean±s.e.m.
Numbers in parentheses are *n* values of surviving mice.
Water consumption values are millilitres of water consumed/mouse per day.

These initial hyperglycaemic levels increased further with duration of disease for diabetic mice in the above groups at 170 and 176 days of age, respectively. In addition, one mouse in the RAPA 0·6 mg/kg group (study no. 2) became diabetic by 135 days and expired at 166 days of age (Fig. 1b). Mice treated with either 6 mg/kg or 12 mg/kg RAPA (study no. 1), 0·6 mg/kg or 6 mg/kg RAPA (study no. 2), maintained normal plasma glucose throughout the experiment.

Water consumption

Water consumption in study nos 1 and 2 increased from baseline (4·3 ml to 4·8 ml water consumed/mouse per day) to peak levels concomitantly with elevated glucose (Figs 1a, b) for naive control (×4·6 increase), vehicle control (×5·2), and RAPA 0·06 mg/kg (×4·6) treated mice. A slightly elevated consumption level reaching 8·8±2·2 ml consumed/mouse per day was observed for the RAPA 0·6 mg/kg group concomitant with the 10% incidence of diabetes. This decreased to baseline levels by 166 days after this mouse expired. In contrast, rapamycin (6 mg/kg or 12 mg/kg, study no. 1; 0·6 mg/kg or 6 mg/kg, study no. 2) treated mice exhibited only a slight increase in water consumption at the end of the studies (×1·4, ×1·7, ×1·2, and ×1·0, respectively) over baseline levels, concomitant with normal weight gain in the mice.

Body weight

Body weights at 8 weeks of age for naive control (20·8 ± 0·5 g), RAPA 6 mg/kg (20·6 ± 0·3 g), RAPA 12 mg/kg (21·1 ± 0·7 g), and at 9 weeks for naive control (22·2 ± 0·4 g), vehicle control (22·2 ± 0·4 g), RAPA 6 mg/kg (22·1 ± 0·4 g), RAPA 0·6 mg/kg (22·0 ± 0·4 g), and RAPA 0·06 mg/kg (22·2 ± 0·5 g) were similar. The non-diabetic mice in all groups gained weight normally and by the end of the study there were no significant differences in per cent change of body weight between rapamycin-treated mice and control mice (study no. 1, 170 days) or between rapamycin-treated mice and vehicle control mice (study no. 2, 176 days). All diabetic mice in the naive, vehicle and RAPA 0·06 mg/kg groups, as well as the single mouse in the RAPA 0·6 mg/kg group, lost weight which varied with the duration of hyperglycaemia (data not shown).

Lipid analysis

In study no. 2, plasma levels of triglyceride, cholesterol, and β-hydroxybutyrate were analysed from the mice at 176 days of age. Although all mice were the same age, their treatment and duration of overt diabetes varied. As is evident from the group means in Table 1, all mice that developed diabetes had elevated levels of plasma triglyceride, cholesterol, and β-hydroxybutyrate. Rapamycin treatment at 0·6 mg/kg and 6 mg/kg in addition to preventing hyperglycaemia (Figs 1a, b) also prevented the associated hyperlipidaemia (Table 1). Furthermore, administration of rapamycin from 0·06 mg/kg to 6 mg/kg did not significantly affect the lipid levels of either the non-diabetic or diabetic mice as compared with the non-diabetic vehicle and diabetic vehicle group, respectively. An examination of individual mice revealed that the plasma levels of β-hydroxybutyrate, triglyceride, and cholesterol were positively correlated ($P < 0·05$) with the duration of hyperglycaemia ($r = 0·85$, 0·87 and 0·84 respectively).

Intervention therapy with rapamycin

Rapamycin administered 6 mg/kg, three times per week p.o., therapeutically to eight NOD mice immediately after IDDM onset at 130–144 days of age was unable to reverse the course of the disease through 176 days of age (data not shown).

Incidence of diabetes after cessation of rapamycin treatment

The 19 non-diabetic mice remaining after 176 days of age from the RAPA 6 mg/kg and RAPA 0·6 mg/kg protected groups were followed for an additional 41-week period after cessation of rapamycin treatment. As shown in Table 2, three of nine mice (33% incidence) from the RAPA 0·6 mg/kg group exhibited delayed onset of IDDM by week 41. As described earlier, there was also a corresponding increase in the mean water consumption for this treatment group which normalized after the three diabetic mice expired. The RAPA 6 mg/kg group continued to have no incidence of diabetes (0/10) with no dipsesis displayed. However, one mouse which had no other clinical signs of IDDM did have a slightly elevated plasma glucose level from 361 days of age through the end of the study at 466 days (Table 2). During this 41-week period, six of the 10 mice previously treated with RAPA (6 mg/kg) expired. Visible tail infections were observed at this time in some mice requiring significant amputation of the remaining tissue. Four of these six deaths may be due to bacterial or viral infection as a result of overimmunosuppression. Necropsy in these mice showed ear infections or white growth present in the chest cavity which contained 'pus' and infiltrated the trachea and lungs. The remaining mice which expired at weeks 35 (425 days of age) and 39 (449 days of age) after cessation of treatment had no visually apparent signs of disease and probably died of natural causes.

DISCUSSION

The present studies show that intermittent oral dosing (three times per week) of rapamycin at concentrations ranging from 0·6 to 12 mg/kg, p.o., prevents the onset of diabetes in NOD mice. Rapamycin-treated mice maintained normal glucose levels throughout the duration of the study and the drug significantly inhibited the progressive water consumption observed in this model. Since water consumption reflects water excreted, the observed normal water consumption of the RAPA-treated mice indicates no osmotic diuresis due to glucosuria. Thus the normal plasma glucose levels observed with RAPA treatment are due to the prevention of diabetes and not due to the elimination of glucose through the urine as is the case for a compound such as phlorizin which lowers plasma glucose by inducing glucosuria [23].

No significant differences in per cent change of body weight were observed in non-diabetic treated mice. In the NOD mice that did become hyperglycaemic, the progression of diabetes followed a typical course of a severe IDDM, i.e. weight loss, and a rise in plasma ketone bodies, triglycerides, and cholesterol [24,25]. The degree of hyperlipidaemia and ketosis was correlated with the duration of uncontrolled hyperglycaemia. Rapamycin-treated mice at both 0·6 mg/kg and 6 mg/kg maintained normal non-diabetic levels of the three plasma lipids. Outside of this ability to prevent diabetes, rapamycin itself did not affect the plasma lipid profile of the mice.

Intervention therapy with 6 mg/kg rapamycin, three times per week p.o., immediately after IDDM was detected, was ineffective at reversing the course of the disease under these experimental conditions. Changes in experimental design are planned to investigate further this parameter as the combination of oral dosing and an intermittent dosing schedule may not have raised rapamycin blood levels to a therapeutic range soon enough to abrogate the attack by T lymphocytes on the pancreatic beta cells. Insulin cotherapy at this stage may also help to 'rest' the remaining beta cells [10,26].

We are encouraged by the level of protection against IDDM demonstrated for both the RAPA 0·6 mg/kg and 6 mg/kg groups in study no. 2, 41 weeks after the cessation of treatment. Our observation of 33% incidence for the RAPA 0·6 mg/kg mice was at reduced levels from expected incidence. Consistent with the findings of Formby *et al.* [2] who reported 25% new incidence of IDDM in NOD mice 5 months (20 weeks) after cessation of CsA treatment, continuous treatment of rapamycin may be needed to prevent IDDM. In this situation, the effectiveness of rapamycin at lower doses using an intermittent dosing schedule may be advantageous in preventing possible complications due to overimmunosuppression. In addition, the administration of insulin prophylactically has been reported to reduce the incidence of development of IDDM in NOD mice [27] and cotherapy with rapamycin may reduce the levels of rapamycin required for prevention. A higher level of rapamycin at 6 mg/kg was able to abrogate the development of IDDM 41 weeks after cessation of treatment. However, since four of 10

mice in this group may have died from complications as a result of overimmunosuppression, we are uncertain whether IDDM would have eventually developed. Our study was not designed to demonstrate the immunological effects of this protection. However, Calne *et al.* [28] and Collier *et al.* [29] have reported that rapamycin induced a state of 'operational tolerance' more than 6 months after treatment ended in three of nine pigs receiving major histoincompatible renal allografts. Morris *et al.* [30] also recently demonstrated that they could induce a state of specific immune unresponsiveness in highly histoincompatible rat heart allografts after cessation of rapamycin treatment. They suggested, among several prospects, the possibility of clonal deletion in their rat model due to the observation of involuted thymi during the rapamycin treatment period. Studies are planned to address these questions.

In conclusion, since the onset of IDDM is becoming more predictable [31], rapamycin may be useful for the prophylactic treatment of autoimmune type I diabetes in man.

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Essential Role for Interferon-γ and Interleukin-6 in Autoimmune Insulin-dependent Diabetes in NOD/Wehi Mice

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Abstract

Experimental studies in vitro suggest that cytokines are important mediators in the pathogenesis of autoimmune insulin-dependent diabetes mellitus (IDDM). However, there is little evidence for the role of cytokines in vivo, either in humans or in the spontaneous animal models of IDDM such as the NOD mouse or BB rat. To address this question, we used the model of cyclophosphamide (CYP)-induced autoimmune diabetes in the NOD/Wehi mouse to examine for (*a*) the production of IFN-γ and IL-6 from isolated islets, and (*b*) the effect of anti IFN-γ or anti IL-6 monoclonal antibodies on the development of diabetes. After cyclophosphamide, the majority of these mice develop of mononuclear cell infiltrate (insulitis) which by 10–14 d is associated with beta cell destruction. IFN-γ activity at low levels (2.7±0.3 U/ml) could be detected only in culture supernatants from islets isolated at day 7 post-cyclophosphamide. In contrast, IL-6 activity progressively increased from 457±44 U/ml at day 0 to 6,020±777 U/ml at day 10. Culture of islets with anti-CD3 monoclonal antibody resulted in a significant increase in IFN-γ activity from 41±7 U/ml at day 0 to 812±156 U/ml at day 10. Mice given either anti-IFN-γ or anti-IL-6 antibody had a significantly reduced ($P < 0.001$) incidence of diabetes and especially with IFN-γ, decreased severity of insulitis. We conclude that IFN-γ and IL-6 have essential roles in the pathogenesis of pancreatic islet beta cell destruction in this model. (*J. Clin. Invest.* 1991. 87:739–742.) Key words: anti-cytokine therapy • pancreatic islet • beta cell

Introduction

A cellular autoimmune process that selectively destroys the pancreatic islet beta cells is thought to be responsible for the development of insulin-dependent diabetes mellitus (IDDM)[1] in humans (1, 2) and in the spontaneous animal models, the BB rat (3) and the NOD mouse (4). A common histopathologic feature associated with the development of IDDM is insulitis, the presence within and around the islets of mononuclear cells consisting predominantly of T lymphocytes and to a lesser degree macrophages (5–7). Experimental strategies aimed at suppressing cellular autoimmunity such as neonatal thymectomy, administration of cyclosporin A or administration of anti-T lymphocyte antibodies prevent development of diabetes (reviewed in references 8 and 9).

Although the molecular pathways for the initiation, perpetuation, and eventual destruction of the beta cell by autoreactive mononuclear cells are unknown, accumulating experimental evidence implicates cytokines as key mediators (reviewed in references 2 and 10). In addition to their actions on immunoinflammatory cells, the cytokines IFN-γ and TNF-α alone or in combination induce, in vitro, the expression of MHC-class I (11, 12), MHC-class II (13, 14) and ICAM-1 (15) molecules by murine and human beta cells. In addition IL-1 (16), IFN-γ, and TNF-α (17, 18) or combinations of these cytokines (17, 18) directly inhibit in vitro, the function and viability of beta cells.

Recently we found that beta cells produce IL-6 and that IL-6 mRNA and protein are markedly increased after exposure of murine islets to IFN-γ and/or TNF-α (19). In view of the wide ranging immunoregulatory effects of IL-6 (reviewed in reference 20), we have hypothesized that its production may confer on the beta cell accessory cell function and thereby perpetuate its immunologic destruction (19).

Apart from experimental studies in vitro there is little evidence that cytokines are present and/or play a role in the pathogenesis of autoimmune beta cell destruction. NOD/Wehi mice develop insulitis but have a low incidence of spontaneous diabetes. However, after a single injection of cyclophosphamide, insulitis is intensified and the majority of mice become diabetic within 2 wk (21). We have used this model to examine for (*a*) the production of IFN-γ and IL-6 from isolated islets, and (*b*) the effect of cytokine neutralization in vivo with anti-IFN-γ and anti-IL-6 antibodies on the development of diabetes.

Methods

Mice. Female NOD/Wehi mice, aged 70–80 d, were used in all experiments. Although insulitis is present in virtually all mice, the incidence of diabetes in female NOD/Wehi mice in contrast to most strains of NOD mice, is < 5% at age 100 d (21). There are no differences between NOD/Wehi and the high-incidence strain, NOD/Lt on the basis of reciprocal skin grafting and allelic enzyme analysis (22).

Monoclonal antibodies and cytokines. Monoclonal antibodies to IFN-γ (RA-642; hybridoma cells obtained from the American Type Culture Collection, Rockville, MD) and IL-6 (6B4; hybridoma cells kindly provided by Dr. J. Van Snick, Brussels, Belgium) were obtained from ascites produced in CBA-Nu/Nu mice. After partial purification

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1. *Abbreviation used in this paper:* IDDM, insulin-dependent diabetes mellitus.

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by NH_4SO_4 precipitation and dialysis, antibodies were diluted to a final concentration of 1 mg/ml in PBS and stored at −70°C until use. Monoclonal antibody to murine CD-3 (145-2C11; reference 23) was a gift from Dr. A. Kelso (Walter and Eliza Hall Institute of Medical Research). Rat immunoglobulin (whole Ig fraction; Jackson Immuno-Research Labs, West Grove, PA) was diluted to 1 mg/ml in PBS before use.

Recombinant murine IFN-γ was a gift from Genentech Inc., South San Francisco, CA, and IL-6 from Dr. J. Van Snick, Brussels, Belgium. The specific activity of these cytokines was 2.3×10^7 U/mg and 1×10^8 U/mg, respectively.

Administration of cyclophosphamide. Euglycaemic mice were injected intraperitoneally on day 0 with 350 mg/kg cyclophosphamide (Cycloblastin, Farmitalia Carlo Erba, Hawthorn, Australia). At this dose some 40–60% of female NOD/Wehi became hyperglycaemic within 14 d.

Pancreatic islet isolation. For cytokine production experiments, islets were isolated by collagenase digestion from overnight fasted mice on days 0, 3, 7, and 10 after cyclophosphamide. Briefly, each pancreas was distended with 10–15 ml of sterile Hepes buffered Kreb's-Ringer bicarbonate buffer, pH 7.5, containing 1 mg/ml BSA and 1 mg/ml D-glucose (HKRB), cut into eight pieces and subsequently placed in sterile vials containing 6 ml of HKRB buffer with 0.8 mg/ml collagenase (type XI; Sigma Chemical Co., St. Louis, MO). After shaking (300 rpm) in an orbital shaker for 25 min at 37°C, digested tissue was washed twice with 10 ml of HKRB buffer, filtered through a sterile 500 μm screen and pelleted by gentle centrifugation (800 $g \times 1$ min). Isolated islets were then purified by discontinuous density-gradient centrifugation through Ficoll (Ficoll-400; Sigma Chemical Co.) at densities of 1.085, 1.075, 1.065, 1.055. Islets present at the 1.075/1.065 and 1.065/1.055 boundaries were further purified by handpicking with the aid of a dissection microscope. This procedure permits the isolation of islets and the associated insulitis lesion, the latter being visible as a clear crescent-shaped mantle surrounding the islet. Groups of 150–200 islets were placed into individual wells of a 24-well cluster plate (Costar, Cambridge, MA) containing 750 μl RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% heat-inactivated FCS (CSL, Parkville, Australia) (RPMI-FCS) and incubated for 72 h. Islets prepared as described above were also cultured in 24-well plates previously coated with CD-3 monoclonal antibody. Purified antibody at 5 μg/ml in PBS was incubated in the wells for 6 h at 37°C, unbound antibody was removed by washing three times in PBS, and coated plates stored at 4°C until use.

Cytokine assays. Assays for IL-6 and IFN-γ were performed using the IL-6 dependent murine plasmacytoma TEPC-2027 (24) and the IFN-γ-sensitive Wehi-279.1 cell lines. TEPC or Wehi cells were seeded in 96-well microtiter plates in RPMI-FCS and cultured at 10^4 cells per well (final volume, 100 μl) in the presence of serial dilutions of culture supernatants or recombinant standard. At day 3 the number of viable cells was measured with the 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl tetrazolium bromide method described by Tada et al. (25) modified as described previously (19). The minimal detection limit was ~ 2 and 5 U/ml for IFN-γ and IL-6, respectively.

Administration of anticytokine antibodies. Euglycemic mice were randomly allocated into three groups of 20 to receive rat Ig, RA-642 or 6B4 in a dose of 0.5 ml (~ 500 μg) on days −1, 3, 6, 9, and 12. Blood samples were collected by orbital sinus bleeding. Blood glucose was measured immediately with B-M glucose test strips (Boehringer-Mannheim, Melbourne, Australia). Mice were considered diabetic when blood glucose was > 15 mmol/liter.

Quantitation of insulitis. Insulitis was scored from the histological appearance of at least 10 islets in a minimum of three sections of each pancreas removed at day 15 and fixed in Bouin's solution. Each islet was scored as to the degree of mononuclear cell infiltration as follows: 0, no infiltration; 1, periductular infiltrate; 2, periislet and periductular infiltrate; 3, intraislet infiltrate; 4, intraislet infiltrate associated with beta cell destruction. The mean score for each pancreas was calculated by dividing the total score by the number of islets scored.

Results

Production of IL-6 and IFN-γ from isolated islets. Supernatants from cultures of NOD islets isolated serially after cyclophosphamide were analyzed for IFN-γ and IL-6 activity (Table I). Low level IFN-γ activity could be detected in culture supernatants from day 7 islets only. In contrast, significant levels of IL-6 activity increased progressively in culture supernatants from islets isolated at day 0 through day 10.

To determine whether or not the low IFN-γ production was due to the absence of functional T cells, isolated islets were cocultured with solid-phase anti-CD3 antibody (Table II). Under these conditions significant IFN-γ activity was detected in the islet supernatants which, in comparison with day 0, decreased at day 3 before increasing significantly at day 7 and 10. Addition of RA-642 antibody completely abolished the IFN-γ activity in day 10 supernatants. Increased IL-6 activity was also present in day 0 and 3 supernatants and further increased to the same level in day 7 and 10 supernatants (Table II). Addition of 6B4 antibody almost completely abolished the IL-6 activity in day 10 supernatants.

Effect of anticytokine antibodies on the development of diabetes. On day 15 after cyclophosphamide administration, 9 of 19 (47%) control mice were diabetic compared with only 1 of 20 (5%) mice treated with anti-IFN-γ antibody and 3 of 19 (16%) mice treated with anti-IL-6 antibody (Table III).

Examination of the pancreas revealed a reduction in the severity of insulitis in mice treated with anticytokine antibody, especially anti-IFN-γ compared with the control mice (Table III).

Discussion

Apart from numerous experimental studies in vitro there has been little direct evidence for the role of cytokines in the pathogenesis of IDDM, either in humans or in the spontaneous animal models. In the present study we used cyclophosphamide-induced autoimmune diabetes in the NOD/Wehi mouse as a model in an attempt to provide this evidence. First, we looked for the production of the cytokines IFN-γ and IL-6 from isolated islets, and second, we examined the therapeutic potential of injected anti-IFN-γ and anti-IL-6 antibodies. The cyclophosphamide model has particular advantages in that the process of autoimmune beta cell destruction is synchronized and the time to onset of hyperglycemia considerably shortened (21). Furthermore cyclophosphamide has been reported not to

Table I. IFN-γ and IL-6 Production from Islets Isolated from Cyclophosphamide-injected NOD/Wehi Mice

	Cytokine activity Day after cyclophosphamide injection			
Cytokine	0	3	7	10
	U/ml			
IFN-γ	ND	ND	2.7±0.3	ND
IL-6	457±44	1136±325	2933±548*	6021±777*

Results are expressed as mean±SEM for triplicate cultures.
ND, not detectable.
* For significance vs. day 0, $P \leq 0.05$ (two-tail Student's t test).

Table II. IFN-γ and IL-6 Production from NOD/Wehi Islets Cultured with Anti-CD3 Antibody

Cytokine	Cytokine activity Day after cyclophosphamide injection					
					10	
	0	3	7	10	+RA-642	+6B4
			U/ml			
IFN-γ	41±13	17±4	378±63*	812±156*	ND	635±92
IL-6	3202±725	2950±700	6188±738*	5494±568*	5386±470	48±9

Results are expressed as mean±SEM for triplicate cultures. ND, not detectable. * Significance vs. day 0, $P < 0.05$ (two-tail Student's t test).

influence beta cell function or viability (26) suggesting that the drug is not directly beta cell toxic. The mechanisms of beta cell destruction in this model are clearly autoimmune and cannot be distinguished from that occurring spontaneously in NOD mice (21, 26). In addition the numbers and phenotype of T lymphocytes infiltrating the pancreas has been defined in this model (27). Therefore, the use of the collagenase digestion procedure which permits the isolation of the intact islet-insulitis lesion makes it possible to study islet-associated T lymphocytes at strategic time points before or after administration of cyclophosphamide.

In the studies with isolated islets barely detectable levels of IFN-γ activity were present and only in supernatants from islets at day 7 postcyclophosphamide. It is not known whether the low level of IFN-γ activity reflects that in vivo in the extracellular milieu or whether T lymphocytes producing IFN-γ are downregulated upon exposure to in vitro culture conditions. Clearly islet-associated T lymphocytes have the potential to produce larger amounts of IFN-γ as evidenced by the results of the experiments in which T lymphocytes were activated via the T cell receptor pathway using an anti-CD3 antibody. In parallel experiments using purified pancreatic T lymphocytes we have observed a qualitatively similar result with significant production of IFN-γ being observed only after stimulation with anti-CD3 antibody (Kay, T., unpublished observation). The IFN-γ activity produced from the unstimulated islet T lymphocytes, although lower than that required in vitro to inhibit beta cell function and viability (17, 18) is within the range for upregulation of beta cell MHC-class I molecules (11) and is therefore biologically relevant.

In contrast to IFN-γ, IL-6 activity was significantly elevated in culture supernatants from islets isolated before cyclophosphamide injection and progressively increased thereafter, indicating a temporal association between production of this cytokine and evolution of the autoimmune lesion. Elevated production of IL-6 has been demonstrated in a number of autoimmune diseases (28). For example, high levels of IL-6 have been found in the synovial fluid of patients with active rheumatoid arthritis (29) and an association between synovial fluid IL-6 and disease activity was reported in patients with inflammatory arthritis of traumatic arthritis (30). The present findings also support the view that the local production of IL-6 reflects disease activity. The fact that stimulated IL-6 production appeared to plateau by day 7 may indicate that a major source of IL-6 is CD4-positive T lymphocytes that predominate at this stage (27). We have reported that beta cells also produce IL-6 and IFN-γ and TNF-α significantly enhance this IL-6 production (19). As has previously been suggested (31) the production of IL-6 induced by cytotoxic cytokines such as IFN-γ and TNF-α or as seen in the present study may constitute part of an injury response by the beta cell that could serve to further enhance the inflammatory response.

Table III. Diabetes Incidence and Degree of Insulitis in Cyclophosphamide-treated NOD/Wehi Mice Given Monoclonal Antibody to Either IFN-γ or IL-6

Antibody	Diabetes, day 15	Insulitis score
Rat Ig (control)	9/19	2.60±0.34
RA-642 (anti-IFN-γ)	1/20*	1.25±0.25‡
6B4 (anti-IL-6)	3/19*	2.10±0.34

* For significance vs. control $P < 0.01$ and $P < 0.05$ (χ^2 test) for HB-170 and 6B4, respectively.
‡ Mean±SEM: significance vs. control, $P < 0.001$ (two-tail Student's t test).

Neutralization of IFN-γ activity in vivo resulted in a pronounced decrease in the incidence of diabetes and was associated with a significant reduction in severity of insulitis, suggesting that anti-IFN-γ treatment is preventative for disease. In contrast to IFN-γ, neutralization of IL-6 activity in vivo while resulting in a reduction in the incidence of diabetes at day 15 was not associated with a significant reduction in insulitis. It is therefore not possible to conclude from the present study whether anti-IL-6 treatment is preventative or delays the onset of diabetes. These differences between IFN-γ and IL-6 may simply reflect the more efficient neutralization of endogenous IFN-γ versus IL-6. Alternatively, IFN-γ may be the more critical cytokine in the initial development of insulitis while IL-6 might be required for subsequent perpetuation, e.g., through enhanced cytotoxic T lymphocyte function (32) of autoimmune beta cell destruction.

In view of their multiple overlapping and cross-regulatory functions (33), it is reasonable to assume that other cytokines, in addition to IFN-γ and IL-6, have a role in the pathogenesis of autoimmune beta cell destruction. As discussed earlier, based on studies in vitro, IL-1 and TNF-α would be good candidates in this role. To our surprise, we have not been able to detect either of these cytokines in supernatants from islets cultured with or without anti-CD3 antibody at any of the time points after cyclophosphamide injection in NOD/Wehi mice (Campbell, I. L., unpublished). In accord with our finding is the recent demonstration that in comparison with SWR mice, peritoneal exudate cells from NOD (high diabetes incidence

strain) mice produce only low levels of TNF-α and IL-1β (34). Therefore the pattern of cytokine expression associated with the development of autoimmune beta cell destruction in the NOD mouse appears to be complex ranging from enhanced production of IFN-γ and IL-6 on the one hand, to deficient production TNF-α and IL-1α on the other.

In conclusion, evidence has been obtained in vivo for an essential role for the cytokines IFN-γ and IL-6 in autoimmune beta cell destruction. Further studies should be directed at defining a composite picture of cytokine expression, and the cellular sources of specific cytokines, during the evolution of insulitis. Our findings are an impetus to the development of anticytokine therapies to prevent beta cell destruction in humans with preclinical IDDM.

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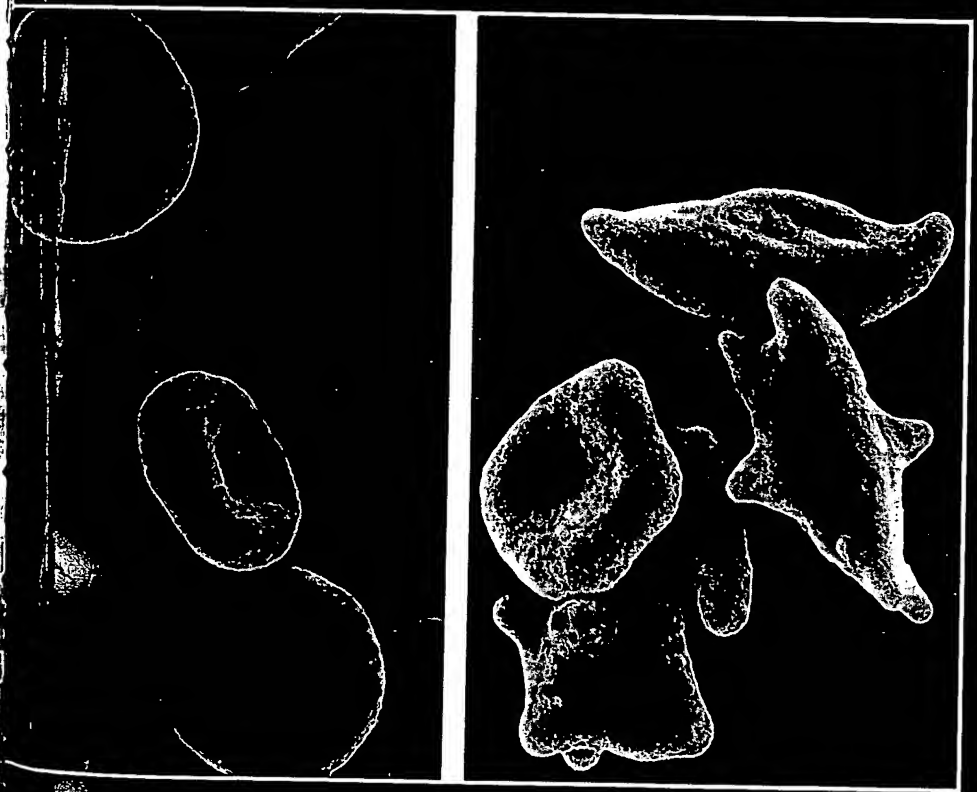

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Interferon-gamma (IFN-γ) production *in vivo* in experimental autoimmune uveoretinitis

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SUMMARY

Experimental autoimmune uveoretinitis (EAU) is a well-characterized model of immune-mediated intraocular inflammation. The intraocular infiltrate in EAU consists predominantly of T lymphocytes. The *in vivo* production of interferon-gamma (IFN-γ) by these T cells was investigated immunohistochemically and by *in situ* hybridization using a cDNA probe to rat IFN-γ mRNA. Positive localization of IFN-γ mRNA began simultaneously with disease onset and increased as the inflammatory tissue destruction progressed. The positive signal was seen on cells in the retina, uveal tract and extraocular region where collections of inflammatory cells contained many T lymphocytes. Numerous cells in these locations also stained positively immunohistochemically for IFN-γ. These results indicate that the *in vivo* production of IFN-γ within the eye could play a role in the immune regulation of intraocular inflammatory disease.

INTRODUCTION

Chronic posterior uveitis and retinal vasculitis are ocular inflammatory conditions which often result in significant visual loss despite treatment. The aetiology of these diseases is largely unknown but autoimmune mechanisms involving ocular antigens have been implicated in their pathogenesis. Experimental autoimmune uveoretinitis (EAU) is an animal model of autoimmune intraocular inflammation[1] induced by systemic immunization with a purified retinal antigen in adjuvant. In the rat model of EAU there is a mixed infiltrate of polymorphonuclear leucocytes and lymphocytes to the anterior and posterior segments of the eye with CD4+ T lymphocytes predominating in the destructive retinal lesions.[2]

EAU can be adoptively transferred by activated, ocular antigen-specific CD4+ T-cell lines[3] demonstrating that these activated T cells are capable of initiating the disease process. The predominant cell infiltrating the retina in the inflammatory phase is also the CD4+ T cell implicating this cell in the destructive inflammatory process.

Murine CD4+ T cells have been divided into two groups based on their lymphokine secretion pattern *in vitro*.[4] Th1 cells produce interleukin-2 (IL-2), interferon-gamma (IFN-γ) and lymphotoxin and are thought to act as primary effector cells. Th2 cells produce IL-4, IL-5 and IL-6 and act as helper cells, for example providing specific B-cell help. Human T-cell clones do not necessarily follow this pattern.[5] The OX22 monoclonal antibody has been used in the rat to characterize CD4+ T cells as naive (OX22 high) and memory (OX22 low).[6] This division appears to have functional significance related to cytokine production, the OX22 high cells producing greater amounts of IL-2 and IFN-γ and the OX22 low cells producing more IL-4.[6-8] Rat uveitogenic T-cell lines have been demonstrated to produce IL-2, IL-4 and IFN-γ when activated *in vitro*[9] but the *in vivo* production of lymphokines in EAU by infiltrating activated T cells is not known.

IFN-γ is a lymphokine produced by activated T lymphocytes and natural killer (NK) cells which has multiple actions in immune response regulation.[10] In addition to its roles in major histocompatibility complex (MHC) class II induction and cellular differentiation and activation it has recently been demonstrated to play an important role in the development of effector cell function in cytolytic T lymphocytes.[11,12] In addition IFN-γ has been shown to induce macrophage activation.[13] IFN-γ is therefore likely to have a key role in the autoimmune inflammatory process.

The aim of this study was to determine if IFN-γ was produced *in vivo* by the infiltrating T lymphocytes in the Lewis rat model of EAU. IFN-γ protein was localized immunohistochemically using a rat IFN-γ-specific monoclonal antibody and a cDNA probe was used to detect IFN-γ mRNA.

MATERIALS AND METHODS

Animals

Female Lewis rats [bred under specific pathogen-free (SPF) conditions, St Thomas's Hospital Medical School, London, U.K.], 100–150 g weight, 6–8 weeks old were used for all experiments.

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Induction of uveitis

Rats were immunized in a hind footpad with 50 μg of purified bovine S-antigen [14] in a 1:1 emulsion in complete Freund's adjuvant (Sigma, Poole, Dorset, U.K.) supplemented with *Mycobacterium tuberculosis* organisms (Sigma) to a final concentration of 1·5 mg/ml. Animals were also given 5×10^9 heat-inactivated *Bordetella pertussis* organisms (Wellcome Laboratories, Beckenham, Kent, U.K.) in 150 μl phosphate-buffered saline (PBS) intraperitoneally. A total of 17 eyes from individual animals were studied. Two eyes from Days 10, 11, 12, 13, and 21, and three eyes from Days 14 and 17 after disease induction, as well as control eyes from a non-immunized animal were removed, embedded in OCT (Shandon, Runcorn, U.K.) and snap frozen in acetone and dry ice. Specimens were stored at −70°.

Probe preparation

A cDNA probe to rat IFN-γ mRNa in the pPC3 plasmid vector was kindly supplied by Dr Ton Kos, TNO, Rijswijk, The Netherlands. The probe consisted of a 528-base pair (bp) fragment comprising two identical repeats of a 264-bp sequence corresponding to the last 21 residues of exon 2, all of exon 3 and the first 60 residues of exon 4 as deduced from the rat IFN-γ gene structure.[15] The plasmid was amplified in HB 101 *Escherichia coli* and the cDNA probe was extracted, purified and labelled with ^{35}S-dCTP alpha (Amersham International, Amersham, Bucks, U.K.) using the random primer technique (Boehringer Mannheim, Lewes, U.K.) and adjusted to 2×10^5 c.p.m./ml in hybridization buffer [600 mM sodium chloride, 50 mM sodium phosphate pH 7·0, 5 M EDTA, 0·02% Ficoll, 0·02% bovine serum albumin, 0·02% polyvinylpyrolidene, 0·1% salmon testis DNA and 50% deionized formamide (all chemicals supplied by Sigma)].

In situ *hybridization*

Whole-eye sections of 12 μm thickness were cut on a cryostat and mounted on specially prepared gelatin (300 bloom swine, Sigma) coated slides. Sections were fixed for 5 min in 4% glutaraldehyde in 0·1 M Sorensens phosphate buffer pH 7·2 with 20% ethylene glycol, rinsed twice in hybridization buffer and soaked in hybridization buffer for 1 hr, rinsed in ethanol and dried. The ^{35}S-labelled probe in hybridization buffer was heated to 90° for 10 min, cooled and 100 μl applied to each slide under a parafilm (Sigma) coverslip. Sections were left to hybridize in a humidified chamber at room temperature for 72 hr. Post-hybridization, slides were immersed in 2 × SSC until the coverslips dislodged, rinsed in 2 × SSC and washed at 40° for 30 min in 1 × SSC. Slides were then rinsed briefly in distilled water, in 70% ethanol for 5 min, in 95% ethanol for 5 min and allowed to dry.

Autoradiography

The slides were dipped in K5 (Ilford, Mobberley, U.K.) photographic emulsion diluted 1:1 in 0·5% glycerol and left to expose at 4° for 21–24 days over silica gel. Slides were then developed for 3·5 min in D19 (Kodak, Hemel Hempstead, U.K.) developer, fixed in Unifix (Kodak), washed in distilled water and counterstained with haematoxylin.

Controls

The control studies outlined below were carried out simultaneously with the *in situ* hybridization.

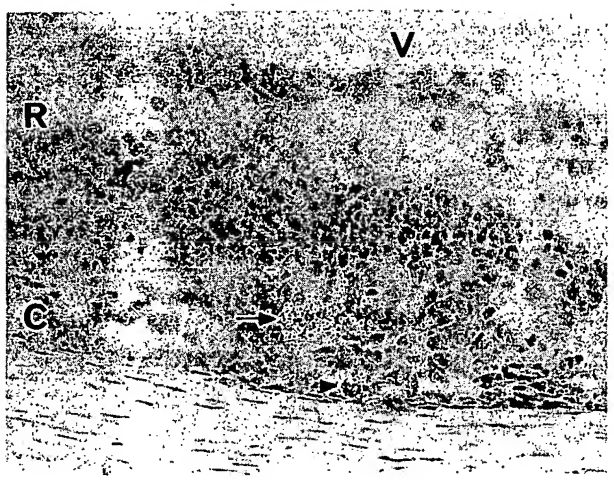

Figure 1. Cells showing positive hybridization signal in the outer retina (arrow) and choroid (arrowhead) at the early stage of inflammation. Retina (R), choroid (C), vitreous (V). Haematoxylin counterstain × 385.

RNAase. Sections from each eye were fixed for 5 min in glutaraldehyde buffer, washed once in 2 × SSC with 5% Tween and three times in 2 × SSC. One hundred microlitres of RNAase A (Boehringer Mannheim) 1 mg/ml in 2 × SSC was applied to each slide under a parafilm coverslip and incubated at 37° for 1 hr, slides were then washed in 2 × SSC and hybridization carried out as above.

P53 probe. To determine the specificity of the IFN-γ probe hybridization, each section was also hybridized with a ^{35}S-labelled probe to the p53 oncogene which was considered to be irrelevant to the EAU disease process.

Non-immunized animal. Eyes from normal, non-immunized Lewis rats were studied both by *in situ* hybridization for IFN-γ mRNA and by immunohistochemistry.

Monoclonal antibodies

For immunohistochemistry primary monoclonal antibodies to rat IFN-γ (Holland Biotechnology, Leiden, The Netherlands), pan T cells (OX19, Serotec, Oxford, U.K.), IL-2 receptor (OX39, Serotec) and MHC class II expression (OX6, Serotec) were utilized.

Immunohistochemistry

Sections of 6 μm thickness were cut from each eye and mounted on 3Amino Propyl Triethoxysilane (APES) (Sigma) coated slides. Slides were fixed in acetone for 7 min and endogenous peroxide activity blocked with 3% hydrogen peroxide in 50% methanol. Sections were stained by a standard avidin-biotin-complex method (Vector, Peterborough, U.K.) using a rat immunoglobulin adsorbed biotinylated secondary antibody (Vector) and amino ethyl carbazole to provide a red final reaction product.

RESULTS

Induction of disease

Animals developed histological evidence of disease on Day 12 post-induction. The histological changes were characteric of rat

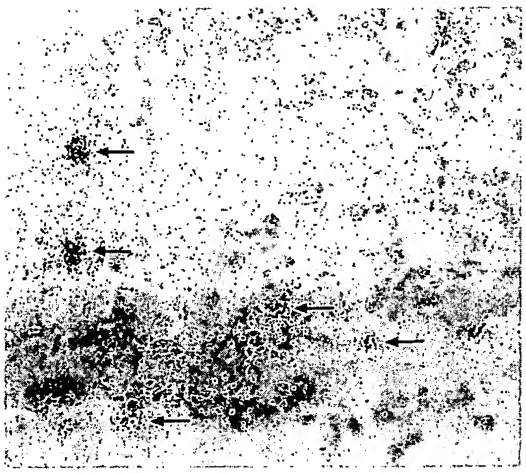

Figure 2. Positive cells (arrows) adjacent to a patch of retinal oedema in early disease. Haematoxylin counterstain ×485.

EAU.[1] There was a marked mixed leucocyte infiltrate of the anterior and posterior segments of the eyes with patches of retinal oedema progressing to total retinal destruction in which lymphocytes were the predominant infiltrating cell.

Localization of IFN-γ mRNA

Positive hybridization signals, observed as collections of dark grains in the photographic emulsion, were seen over cells from Day 12 post-immunization onwards. In the early stages of the disease process positive cells were seen in the outer retina and choroid (Fig. 1) and related to patches of retinal destruction and oedema (Fig. 2). As the retinal destructive process progressed increasing numbers of positive cells were seen in these regions (Fig. 3a) as well as in accumulations of inflammatory cells in the ciliary body and the anterior extraocular region. Later in the disease when there was marked retinal destruction, positive cells were found predominantly in the choroid.

Immunohistochemical localization of IFN-γ

The IFN-γ monoclonal antibody gave positive membrane staining of cells from Day 12 post-induction onwards. Mononuclear cells in the extraocular tissues, anterior chamber, ciliary body, choroid, retina (Fig. 4), vitreal cavity and subretinal fluid all showed positive staining. The staining intensity increased as the inflammatory process progressed.

Cellular immunohistochemistry

OX19 (pan T cell) positive cells were found in inflammatory infiltrates throughout the extraocular tissues, anterior chamber, ciliary body, choroid and retina, including the areas of IFN-γ mRNA localization (not shown). Cells in these regions also showed positive staining with the OX39 antibody for IL-2 receptor expression. MHC class II expression was found on cells from Day 10 post-induction onwards; this increased markedly as the disease progressed and was seen both on organ-resident and infiltrating cells (not shown).

Controls

The eyes from non-immunized animals showed no localization of IFN-γ mRNA or evidence of infiltrating inflammatory cells. Pre-treatment with RNAase abolished all localizing hybridization signal (Fig. 3b) and hybridization with the p53 oncogene probe gave only a vague background signal in the inflamed tissue without any cellular localization (not shown).

DISCUSSION

We have demonstrated the presence of IFN-γ mRNA localized to cells in areas of T-lymphocyte infiltration in the rat model of experimental autoimmune uveoretinitis. Furthermore we have shown that IFN-γ is present throughout these eyes using a monoclonal antibody specific for rat IFN-γ. These results correlate with the immunohistochemical finding of IFN-γ related to areas of activated T-cell infiltration in human posterior uveitis.[16] IFN-γ mRNA was found in two areas within the eyes of rats with EAU, the retina and the uveal tract (choroid and ciliary body) and also in extraocular inflammatory cell infiltrates. No IFN-γ mRNA was found in cells in the anterior chamber or vitreous cavity although many T lymphocytes were present in these sites. This would imply that IFN-γ production takes place in the retina, choroid or ciliary body where activation of the T lymphocytes may take place or in the extraocular region where cells are trafficking to and from the eye. It follows that once T cells are in the anterior chamber or vitreous cavities they no longer produce IFN-γ.

Greater numbers of T cells had strong immunohistochemical staining for IFN-γ than had a positive signal for IFN-γ mRNA. It is likely that this is due to the transient expression of mRNA for this cytokine by T cells[8] compared to a longer time period when cytokine protein can be demonstrated on cells, either localized to cytokine receptors or on cells no longer expressing cytokine mRNA.

It has recently been demonstrated that systemically administered IFN-γ down-regulates the immune response in the mouse model of EAU.[17] The demonstration that there is increased mRNA expression for IFN-γ in the later stages of the intaocular destructive process is evidence that its local production could play a role in the immune regulation of this disease *in vivo*.

IFN-γ is known to induce or up-regulate MHC class II antigen expression on a variety of cell types.[10] It has been shown that IFN-γ can induce MHC class II expression *in vitro* in rat, guinea-pig[18] and human[19] retinal pigment epithelial (RPE) cells, a layer of cells lying between retina and choroid forming part of the blood-retinal barrier. RPE cells are known to express MHC class II antigens *in vivo* in human posterior uveitis[20] and in EAU[21] and it has been proposed that such aberrant expression of class II may be important in the development of autoimmunity.[22] Local production of IFN-γ provides a mechanism whereby aberrant expression of class II antigens on organ resident cells can occur in uveitis.

Intraocular injection of recombinant IFN-γ into the vitreous cavity of rat eyes has been demonstrated to induce *in vivo* MHC class II expression on organ-resident cells in the uveal tract and cornea and also on extraocular conjunctival epithelial cells. Subretinal injection of recombinant IFN-γ produces *in vivo* class II expression on RPE cells in rats.[23] These changes were shown to be associated with an infiltrate of both neutrophil polymorphonuclear leucocytes and monocytes to the iris and inner

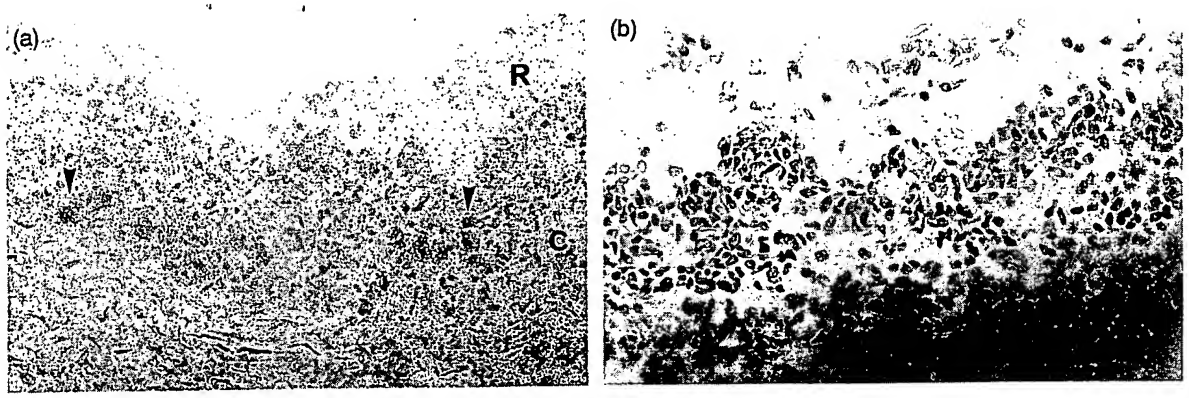

Figure 3. (a) Numerous positive cells (arrowheads to examples) within disorganized retina (R) and choroid (C) in advanced inflammation. Haematoxylin counterstain ×300. (b) Retina from adjacent section to (a) pre-treated with RNAase. No localizing hybridization signal. Haematoxylin counterstain ×300.

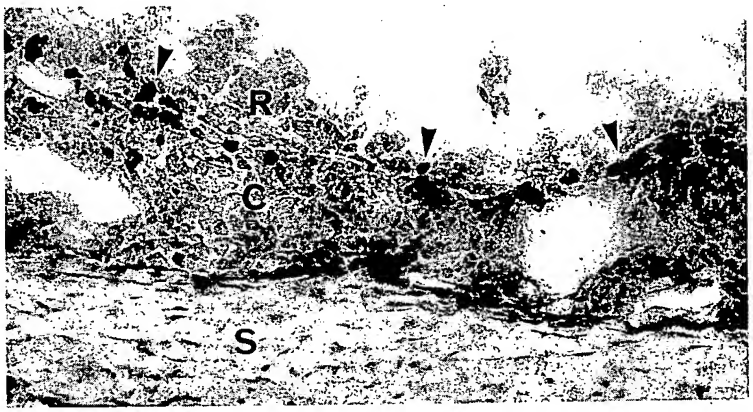

Figure 4. Immunohistochemical localization of cells staining positively for IFN-γ within the retina (R) and choroid (C) at a stage of advanced destruction (arrowheads to examples). Sclera (S). Haematoxylin counterstain ×300.

retinal layers suggesting that local IFN-γ plays an important role in the induction of autoimmune inflammatory eye disease. This finding is contrary to the aforementioned down-regulation of murine EAU by systemic IFN-γ[17] and the exact role of locally produced IFN-γ in EAU requires further elucidation.

The investigation of patterns of cytokine production has given information on the roles of T-cell subsets in the mouse,[4] rat[6,7,8,24] and in humans.[5,7,25] The murine subgroups Th1 and Th2 do not appear to be consistent in rats and humans. IFN-γ is produced predominantly by the OX22 high subgroup of rat $CD4^+$ T cells which are thought to represent naive cells prior to antigen contact.[7,24] This finding is in conflict with human $CD4^+$ T cells where CD45RO (memory) T cells produce IFN-γ. It has been proposed that a further subset of human $CD4^+$ T cells may exist and that rat and human $CD4^+$ T cells may therefore have similar patterns of lymphokine secretion.[7] Analysis of rat $CD4^+$ T cells for IFN-γ mRNA has shown that there is a greater frequency of positive cells in the OX22 low group although the level of secreted IFN-γ protein is higher from OX22 high cells.[8] This may be due to post transcriptional regulation of translation of IFN-γ mRNA or regulation of secretion of IFN-γ protein since continued stimulation of OX22 low T cells results in a down-regulation of expression of IFN-γ mRNA. The IFN-γ mRNA expressing cells found in EAU may be from either the OX22 high or low groups and further analysis of the cells infiltrating the retina in EAU by flow cytometry would be necessary to determine their exact phenotype.

Although the identity of the effector cells in the destructive retinal lesions in EAU is not known, T lymphocytes and macrophages have been shown to be present in large numbers in these lesions as the disease progresses.[1] IFN-γ is capable of producing development of cytotoxic T-lymphocyte precursors into cytotoxic effector cells[11,12] and also has the potential to activate macrophages,[13] its *in situ* production at the sites of retinal destruction in EAU would potentially enable both these cell types to act as effector cells. The further investigation of the pattern of cytokine production in these destructive foci will help us to understand the roles of these cells in the disease process.

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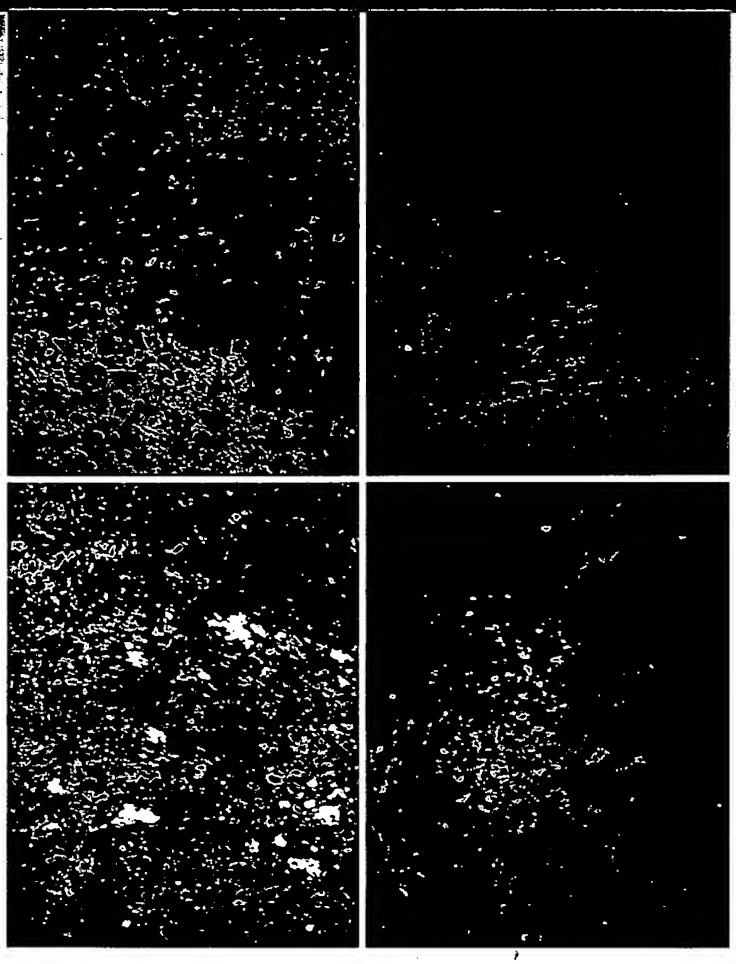

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PREVENTION OF INSULITIS AND DIABETES IN NONOBESE DIABETIC MICE BY ADMINISTRATION OF FK506[1]

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We investigated the preventive effect of the immunosuppressive agent FK506 on autoimmune insulitis in nonobese diabetic mice. The mice were given FK506 in a dose of 1.0 mg/kg, every other day, from age 2 to 12, 2 to 6, and 4 to 12 weeks, respectively; after which, the incidence of insulitis and overt diabetes was monitored. Effects of FK506 on immune reactions to beta cells were also investigated by using both syngeneic and allogeneic islet transplants. Treatment with FK506 in mice from age 2 weeks prevented completely the onset of overt diabetes, and the incidence of insulitis was reduced to less than 10% at age 30 weeks. Treatment of mice with FK506 from age 4 weeks was less effective in preventing insulitis and the onset of diabetes. In case of islet transplantation, FK506 treatment of NOD mice from age 2 to 6 weeks prevented autoimmune responses both in syngeneic islets and in allogeneic islets, which share the same H-2 antigen with the nonobese diabetic mouse. These results also indicate that the recognition of islet antigens and the generation of autoimmune-reactive T lymphocytes start between 2 and 4 weeks of age, and FK506 prevents an autoimmune reaction.

In nonobese diabetic (NOD)* mice diabetes develops spontaneously as a result of an autoimmune response to beta cells in the pancreatic islets. This has served as a model of type 1 diabetes in humans (*1, 2*). Studies on islet transplantation in these NOD mice showed that syngeneic islets transplanted into the subcapsular space of the kidney were immediately rejected, even with cyclosporine treatment (*3, 4*). Reasons for this unsuccessful transplantation are considered to be as follows: (1) immunological recognition of the islets in this strain was already established when the immunosuppressive agent was initiated; and (2) CsA did not suppress the autoimmunological reaction to the transplanted islets.

We previously reported that treatment with FK506 from age 2 to 6 weeks prevented the onset of overt diabetes in NOD mice, in which the earliest onset of positive glucosuria occurred at 12 weeks in normal condition. This finding may suggest that autoimmune priming directed against beta cells takes place as early as 2 weeks of age, and that FK506 has a preventive effect on autoimmune reactions administered prior to this immune priming (*5*). The present study was carried out to further confirm our hypothesis that immunosuppressive treatment prior to the occurrence of immunological recognition would be effective in preventing autoimmune-related destruction. For this purpose, an immunosuppressive agent was administered to NOD mice of different ages, and evaluations were made on the effects in syngeneic and allogeneic models of islet transplantation. The immunosuppressive agent, FK506, which we used was isolated from a Streptomyces in 1984 (*6*), and functions to suppress the secretion of lymphokines from T lymphocytes and to inhibit the induction of allospecific cytolytic T lymphocytes (CTL) during recognition and induction phases of the allograft reaction (*7–9*).

MATERIALS AND METHODS

Animals. NOD(H-2K^d,D^b), BALB/c(H-2^d), and B10.AKM(H-2K^k,D^d) female mice purchased from Clea Co., Ltd. (Tokyo, Japan) and were bred under pathogen-free conditions in the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine. In the NOD mice, preliminary studies showed that the earliest onset of positive glucosuria occurred at age 12 weeks; therefore, urinary glucose levels were tested once weekly in mice from age 10 to 15 and every other day from age 15 weeks. NOD mice with a persistent positive glucosuria were defined as cases of overt diabetes and were given daily injections of insulin (Lente MC, Novo-Nordisk A/S, Denmark). The cumulative incidence of diabetes in our colony was about 75% at age 30 weeks in the female NOD mice.

Immunosuppressive agent. FK506 (Lot. 116393K), kindly provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan), was suspended in normal saline at a concentration of 0.2 mg/ml and kept at 4°C. In preliminary experiments, FK506 in a dose of 1.0 mg/kg body weight proved adequate for allogeneic skin transplantation in diabetic NOD mice, and 1.0 mg/kg of FK506 was given to mice used in the present study.

Treatment protocols for FK506 and monitoring diabetes and insulitis in NOD mice. FK506 was given intraperitoneally to NOD mice every other day. The experiment included the following four groups. Group 1: FK506 treatment started at age 4 weeks and continued until 12 weeks (n=15); group 2: FK506 treatment started at age 2 weeks and continued until 12 weeks (n=15); group 3: FK506 treatment started at age 2 weeks and continued until 6 weeks (n=15); and group 4: no treatment (n=20). In each group, the cumulative incidence of overt diabetes was evaluated at age 30 weeks. In addition, 5 mice in each group were killed at age 6, 12, 20, and 30 weeks, respectively, for histological evaluations of the pancreas, and these mice were excluded from the above experiment in number. The pancreatic tissue was fixed in 10% formalin, serial sections were cut and stained with hematoxylin and eosin, and the degree of insulitis was assessed. Fifty different islets from each pancreas were microscopically examined. Insulitis was defined as positive when mononuclear cell infiltration was evident. The percentage of islets with insulitis was calculated, and results were compared among the four groups.

Transplantation of the neonate pancreas. To investigate preventive effects of FK506 in syngeneic and allogeneic islet transplantation, pancreata were removed from NOD mice within 3 days of birth and were minced into 0.5–1.0-mm^3 pieces in Hank's solution. These tissues were then transplanted into the subcapsular space of the left kidney of 20-week-old female NOD mice, separated into three groups: group A, recipients were not treated with FK506 (n=5); group B, treated with

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* Abbreviatons: CTL, cytolytic T lymphocytes; MLR, mixed lymphocyte reaction; NOD, nonobese diabetic.

FK506 for 10 days beginning on the day of transplantation; group C, treated with FK506 from age 2 to 6 weeks (n=6). The mouse was killed on the 10th posttransplant day, and the graft and kidney were fixed in 10% formalin until histological evaluations were made. Intensity of the lymphocytic infiltration was graded in the following manner: (−) no or little lymphocytic infiltration with normal islets; (+) a moderate infiltration to the islets in the grafts; and (++) a severe infiltration covering the grafts. Identification of beta cells in the graft was determined using anti-insulin antibody and an avidin-biotin-peroxidase complex method (*10*).

In the subsequent experiment, allogeneic pancreata from BALB/c or B10.AKM newborn mice were transplanted into NOD recipients. Recipients were 20-week-old female NOD mice separated into the following five groups: group I, recipients received BALB/c pancreata tissues and were not treated with FK506 (n=5); group II, recipients received BALB/c pancreata tissues and were treated with FK506 for 10 days beginning on the day of transplantation (n=5); group III, recipients received BALB/c pancreata tissues and were treated with FK506 from age 2 to 6 weeks and for 10 days beginning on the day of transplantation (n=5); group IV, recipients received B10.AKM pancreata tissues and were not treated with FK506 (n=3); and group V, recipients received B10.AKM pancreata tissues and were treated with FK506 for 10 days beginning on the day of transplantation (n=3). Ten days after transplantation the grafts were histologically examined.

Mixed lymphocyte reaction (MLR). MLR was performed to determine whether or not the mice treated with FK506 maintained their response to allolymphocytes. Responder cells were prepared from the spleen of 13-week-old NOD ($H\text{-}2K^d,D^b$) mice treated with FK506 from age 2 to 12 weeks. Stimulator cells prepared from the spleen of BALB/c($H\text{-}2^d$) and C57BL/6($H\text{-}2^b$) mice were treated with mitomycin C (Kyowa Hakko Kogyo, Tokyo); 3×10^4 responder cells and 2×10^4 stimulator cells were incubated in 96-well microtiter plates for 5 days at 37°C, then ^{3}H-thymidine was added, the cultures were incubated for 12 hr, and the radioactivity was assessed using a liquid scintillation counter.

Lymphocyte subsets analysis. Spleen cells from 20-week-old NOD mice, those not treated, and those treated with FK506 from age 2 to 6 weeks, were prepared for analyses of phenotypic lymphocyte subsets. Fluorescein isothiocyanate–conjugated rat anti-Thy1.2 monoclonal antibody, FITC-conjugated rat anti-Lyt2 MoAb, and phycoerythrin-conjugated rat anti-L3T4 MoAb (Becton Dickinson) were used for the phenotypic population of pan-T-cells, $CD8^+$ cells, and $CD4^+$ cells, respectively. The samples were analyzed on a FACScan. Those MoAbs and FACScan were all obtained from (Becton Dickinson, Mountain View, CA).

Statistical analyses. The chi-square test was used for comparison of the cumulative incidence of diabetes, and Student's *t* test was used for comparison of the degree of insulitis and for a comparison of lymphocyte subsets. A *P* value less than 0.05 was considered to have statistical significance.

RESULTS

Preventive effect of FK506 on insulitis and overt diabetes in NOD mice. At age 30 weeks, the cumulative incidence of diabetes in the control group (group 4) was 70% (14/20) while the cumulative incidences of diabetes in groups 1, 2, and 3 were 6.7% (1/15, $P<0.01$); 0% (0/15, $P<0.01$); and 0% (0/15, $P<0.01$), respectively (Table 1).

Histologically, the incidence of insulitis in group 1 was 1±2, 57±38, and 36±27% (n=5, means ±SD) at age 12, 20, and 30 weeks, respectively. In group 2, it was 0, 0, and 7±2% at age 12, 20, and 30 weeks, respectively. In group 3, the incidence was 0, 0, 3±6, and 3±1% at age 6, 12, 20, and 30 weeks, respectively; and in group 4, it was 31±13, 55±7, 80±14, and 80±20% at age 6, 12, 20, and 30 weeks, respectively (Table 2).

Syngeneic pancreas transplantation. In group A, where none of the recipients were treated with FK506, grafts showed severe mononuclear cell infiltration, and a few islets were present in all five mice. In group B, all the recipients were treated with FK506 after transplantation, and the grafts showed mononuclear cell infiltration and disappearance of islets in three of five mice, while in the other two mice the grafts showed some islets with lymphocytic infiltration (Figs. 1 and 2). In group C, all the recipients were treated with FK506 from age 2 to 6 weeks, and their grafts demonstrated little mononuclear cell infiltration (Fig. 3) and included insulin-positive cells (Fig. 4) (Table 3).

TABLE 1. The cumulative incidence of diabetes in NOD mice[a]

Groups	Treatment with FK506	Overt diabetes
1 (n=15)	4–12 Weeks of age	1 (6.7%)[b]
2 (n=15)	2–12 Weeks of age	0 (0.0%)[b]
3 (n=15)	2–6 Weeks of age	0 (0.0%)[b]
4 (n=20)	(−)	14 (70.0%)

[a] The cumulative incidence of diabetes in the control group was 70% at 30 weeks of age while that in groups 1, 2, and 3 was 6.7%, 0, and 0, respectively (NOD: nonobese diabetic).
[b] Significant difference from group 4; $P<0.01$.

TABLE 2. The incidence of insulitis[a]

Groups	NOD[b] mice, weeks of age			
	6 Weeks	12 Weeks	20 Weeks	30 Weeks
1		1±2%[c]	57±38%	36±27%
2		0%[c]	0%[c]	7±2%[c]
3	0%[c]	0%[c]	3±6%[c]	3±1%[c]
4	31±13%	55±7%	80±14%	80±20%

[a] Treatment with FK506 from age 2 weeks (groups 2 and 3) prevented the development of insulitis, while when treatment was begun from age 4 weeks (group 1) the effects were less favorable. Values are means ± SD (n=5).
[b] NOD: nonobese diabetic.
[c] Significant difference from group 4; $P<0.01$.

Allogeneic pancreas transplantation. In group I, none of the mice were treated with FK506, and all grafts had a severe lymphocytic infiltration and no islets. In group II treated with FK506 after transplantation, the grafts had lymphocytic infiltration to islets, but this was not so severe as in group I. In group III treated with FK506 from age 2 to 6 weeks and after transplantation, the grafts showed islets with little lymphocytic infiltration. In group IV, the recipients received B10.AKM pancreata tissues and were not treated with FK506. In this group, the grafts showed severe lymphocytic infiltration and no islets. In group V, the recipients received B10.AKM pancreata tissues and were treated with FK506 after transplantation. Their grafts showed intact islets and no lymphocytic infiltration (Table 4).

Adverse effects of FK506. In the first experiment, most of the mice treated with FK506 from age 2 weeks gained little weight in the early stages of treatment, and about 10% of the mice died by age 6 weeks. The other mice gained weight by age 10 weeks. The mice treated from age 4 weeks did not lose weight in the early stages of treatment. Four mice treated with FK506 from age 2 to 12 weeks, one treated from age 2 to 6 weeks, and one treated from age 4 to 12 weeks lost weight and died at an age of 10–20 weeks. Autopsy revealed a large thymoma associated with bilateral hydrothorax in all of six mice. These findings were absent in the controls. In examinations of pancreas trans-

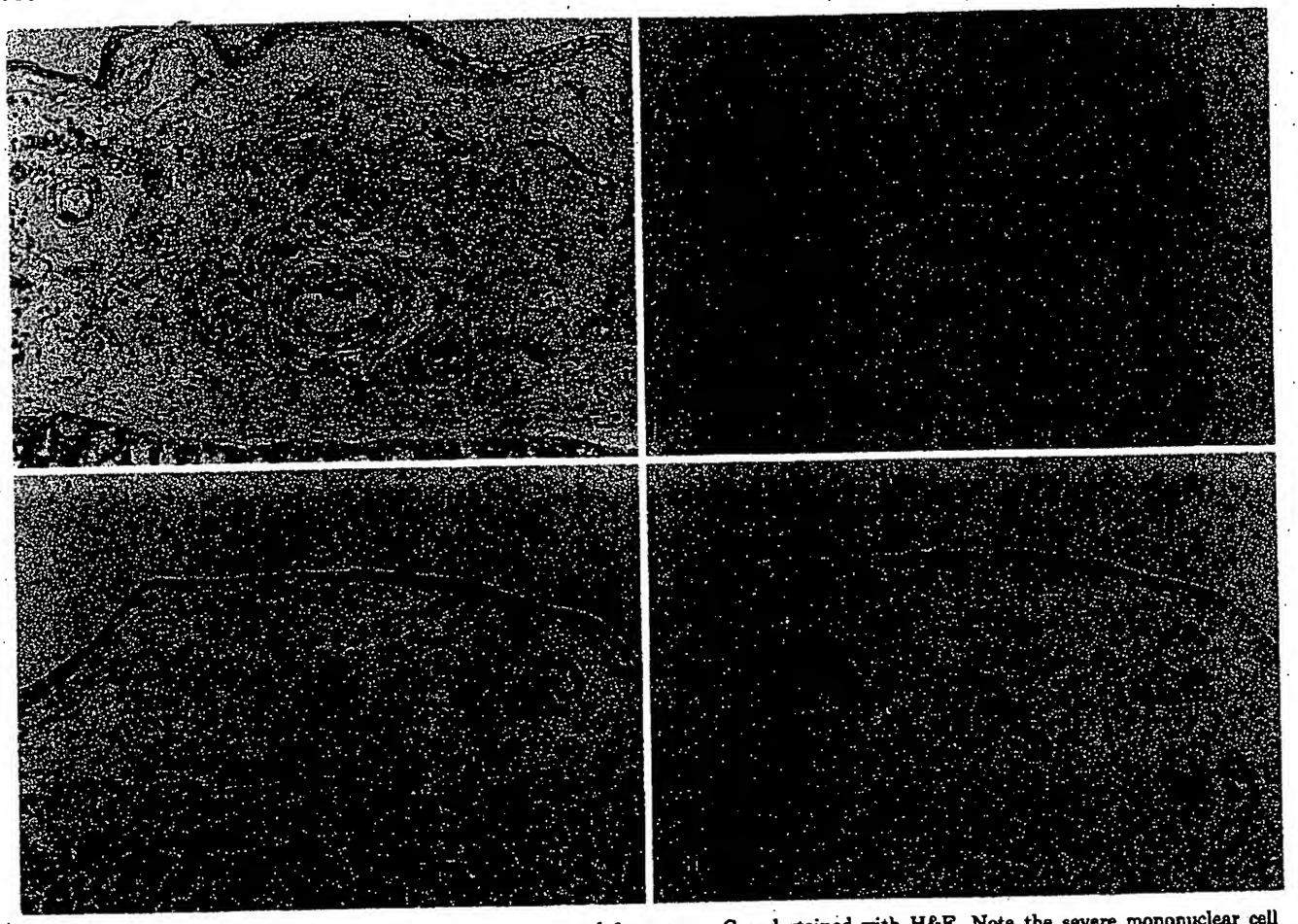

FIGURE 1. Ten days after transplantation, grafts are removed from group C and stained with H&E. Note the severe mononuclear cell infiltration into the iselts (×200).

FIGURE 2. The same graft as in Figure 1 is stained with the indirect avidin-biotin complex method to identify insulin-producing cells. The graft demonstrates a few insulin-producing cells within the severe infiltration of mononuclear cells (×200).

FIGURE 3. A graft stained with H&E in group B reveals intact islets with little mononuclear cell infiltration (×200).

FIGURE 4. The same graft as in Figure 3 demonstrates insulin-producing cells identified by antiinsulin antibody and the indirect avidin-biotin complex method (×200).

TABLE 3. Syngeneic pancreas transplantation

Groups[a]	Recipient	Donor	Treatment with FK506	Infiltration
A (n=5)	NOD[b]	NOD	(−)	(++)
B (n=5)	NOD	NOD	After transplantation	(+)
C (n=5)	NOD	NOD	2–6 weeks of age	(−)

[a] In groups A and B, grafts showed lymphocytic infiltration, and in group C the grafts had normal islets and no lymphocytic infiltration.
[b] NOD: nonobese diabetic.

TABLE 4. Allogeneic pancreas transplantation[a]

Groups	Recipient	Donor	Treatment with FK506	Infiltration
I (n=5)	NOD[b]	BALB/c	(−)[c]	(++)[d]
II (n=5)	NOD	BALB/c	After transplantation	(+),[e] (++)[d]
III (n=5)	NOD	BALB/c	2–6 weeks of age and after transplantation	(−),[c] (+)[e]
IV (n=3)	NOD	B10.AKM	(−)[c]	(++)[d]
V (n=3)	NOD	B10.AKM	After transplantation	(−)[c]

[a] Treatment with FK506 following transplantation did not protect the BALB/c grafts sharing H-2K^d antigen from lymphocytic infiltration while this treatment protected the B10.AKM grafts, incompatible with MHC loci, from lymphocytic infiltration.
[b] NOD: nonobese diabetic.
[c] (−): None or little lymphocytic infiltration.
[d] (++): Severe infiltration covering the graft.
[e] (+): Moderate infiltration into islets in the grafts.

plantation, the recipient mice treated with FK506 1 mg/kg/day showed no particular adverse effects.

Lymphocyte subsets analyses. In analyses of lymphocyte subsets, the frequency of L3T4-positive, Lyt2-positive, and Thy1.2-positive cells in the control group as compared with the FK506-treated group was 35.7±4.9% vs. 23.6±5.8% ($P<0.01$); 10.5±2.4% vs. 7.7±1.8%; and 29.5±3.9% vs. 34.2±2.1%, respectively (Fig. 5). Only the population of L3T4-positive lymphocytes significantly decreased in NOD mice given FK506.

MLR. Spleen cells from 13-week-old NOD mice given FK506 from age 2 to 12 weeks showed a proliferative response to allolymphocytes but no response to splenic lymphocytes prepared from control NOD mice ($P<0.01$), as shown in Figure 6.

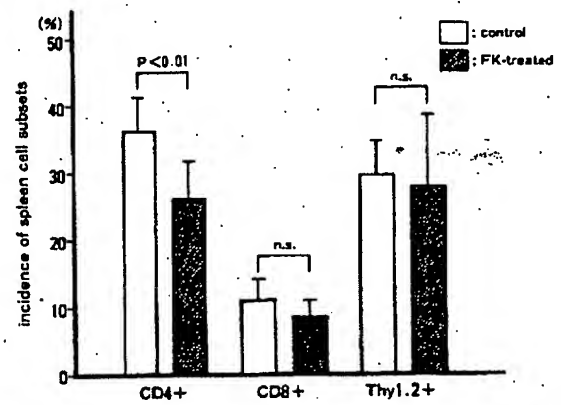

FIGURE 5. The percentage of fluorescein-positive cells from spleen cells of NOD mice at 13 weeks of age. Open bars: control NOD mice (n=5); shaded bars: FK506-treated NOD mice (n=4); $CD4^+$: CD4-positive cells; $CD8^+$: CD8-positive cells; Thy1.2: Thy1.2-positive cells (values are means ±SD; n.s.: not significant).

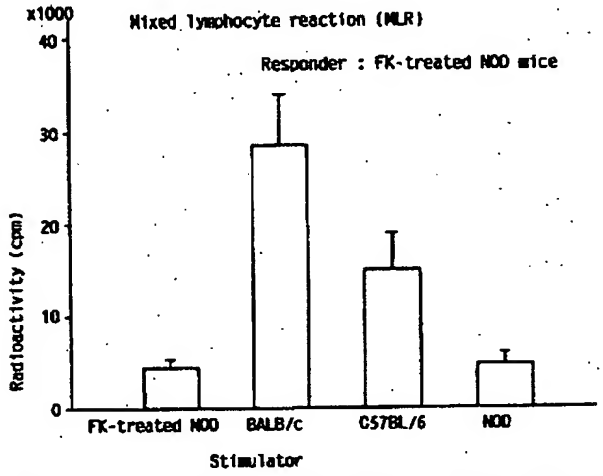

FIGURE 6. Splenic cells from NOD mice treated with FK506 from 2 to 6 weeks of age showed a proliferative response to allolymphocytes in MLR.

DISCUSSION

It has been documented that T lymphocytes (*2, 11, 12*) and macrophages (*13*) play an important role in initiating insulitis. There is also evidence that immunosuppressive agents such as CsA (*14*) and FK506 (*15, 16*) exert preventive effects when given to young NOD mice. As previously reported, treatment with FK506 from age 4 to 12 weeks significantly suppressed the onset of overt diabetes and insulitis in NOD mice; however, administration of the drug from age 2 to 12 weeks was even more effective in preventing diabetes and insulitis. Short-term treatment with FK506 from age 2 to 6 weeks also completely prevented the onset of overt diabetes and significantly reduced the degree of insulitis (*5*). Thus FK506 should be started at age 2–4 weeks.

Although FK506 does not inhibit the proliferation of activated T lymphocytes, it does suppress the release of IL-2 and gamma-interferon from alloprimed T lymphocytes (*7–9*). Therefore this immunosuppressant is thought to have an immunosuppressive effect during recognition and induction phases of allotransplantation. These immunosuppressive effects are about 100 times greater than those induced by CsA (*6*).

In cases of syngeneic islet transplantation, grafts in mice treated with FK506 from age 2 to 6 weeks were not rejected. These findings suggest that the occurrence of autoimmunological recognition and generation of CTL against their own islets began from age 2 weeks. In experiments involving allogeneic pancreas transplantation into NOD recipients, treatment with FK506 following transplantation protected the B10.AKM grafts, incompatible at all major histocompatibility complex loci with NOD, from lymphocytic infiltration, and from destruction of the beta cells. However, this posttransplant treatment with FK506 did not protect the BALB/c grafts sharing $H\text{-}2K^d$ antigen from severe lymphocytic infiltration or destruction of beta cells. Therefore, posttransplant treatment with FK506 at 1.0 mg/kg did not suppress the autoimmune response to beta cells, which exerted its effect in an H-2-restricted manner (*4*). Recipients treated with FK506 from age 2 weeks did not reject the BALB/c grafts, findings consistent with our results regarding the occurrence of autoimmune recognition to beta cells in NOD mice. To prevent this autoimmune response that reacts in an H-2-restricted manner, FK506 treatment needs to be initiated prior to the occurrence of autoimmunity, from age 2 weeks.

In the subpopulation of splenic mononuclear cells, in mice treated with FK506 from age 2 to 6 weeks, there was a significant decrease in CD4-positive cells. With regard to CD8-positive cells, Thy1.2-positive cells, and CD4/CD8 ratio, there was no significant difference between the control group and the FK506-treated group.

In MLR, spleen cells from mice treated with FK506 showed a proliferative response to allolymphocytes, thus treatment with FK506 did not suppress the immune system nonspecifically, but rather it may induce peripheral tolerance in the islets (beta cells). Additional studies on the effects of FK506 in vivo and in vitro are needed to elucidate the mechanism of this induction of tolerance.

The main new findings in our experiments are: (1) FK506 prevents autoimmune reaction; and (2) this effect can be induced by short-term treatment prior to autoimmunity in NOD mice. These findings may provide the most effective method of immunosuppression in autoimmune diseases.

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RAPID COMMUNICATION

USE OF DONOR β_2-MICROGLOBULIN-DEFICIENT TRANSGENIC MOUSE LIVER CELLS FOR ISOGRAFTS, ALLOGRAFTS, AND XENOGRAFTS[1]

XIANGPING LI AND DENISE FAUSTMAN[2]

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Donor graft major histocompatibility complex class I antigens are targets for both allogeneic and xenogeneic rejection. Mice homozygous for β_2-microglobulin gene disruption express reduced amounts of surface MHC class I antigens. Liver cells from such mutant mice were transplanted into isogeneic, allogeneic, and xenogeneic recipients to evaluate the potential of these animals as transplant donors. The survival of allografts of transgenic 129 mouse liver cells in 15 immunocompetent and histoincompatible mouse recipients (BALB/c, D1.C) was only slightly improved 30 days after transplantation relative to normal 129 mouse allografts. These results could be attributable: (1) to host natural killer cell-mediated lysis of donor MHC class I antigen-deficient cells; (2) to donor liver cell MHC class I determinants that are reduced but not eliminated serving as rejection targets; (3) to the plentiful host supply of serum β_2-microglobulin reconstituting the graft and restoring donor MHC class I. Culture studies confirmed the ability of exogenous human and bovine β_2-microglobulin to restore rapidly MHC class I antigen expression on transgenic cells. Because cell surface exchange of β_2-microglobulin is less efficient between species with divergent β_2-microglobulin sequences, the survival of transgenic 129 mouse liver cells in guinea pig (60% β_2-microglobulin identity) and *Xenopus* (34% β_2-microglobulin identity) hosts was investigated. Significant prolongation of MHC class I antigen-deficient liver cell xenografts was apparently only in *Xenopus* hosts. Furthermore, transplants of transgenic 129 mouse liver cells into isogeneic normal 129 mouse recipients showed evidence of rejection in seven of nine recipients, suggesting that transgenic donor cells also may be susceptible to lysis by host natural killer cells.

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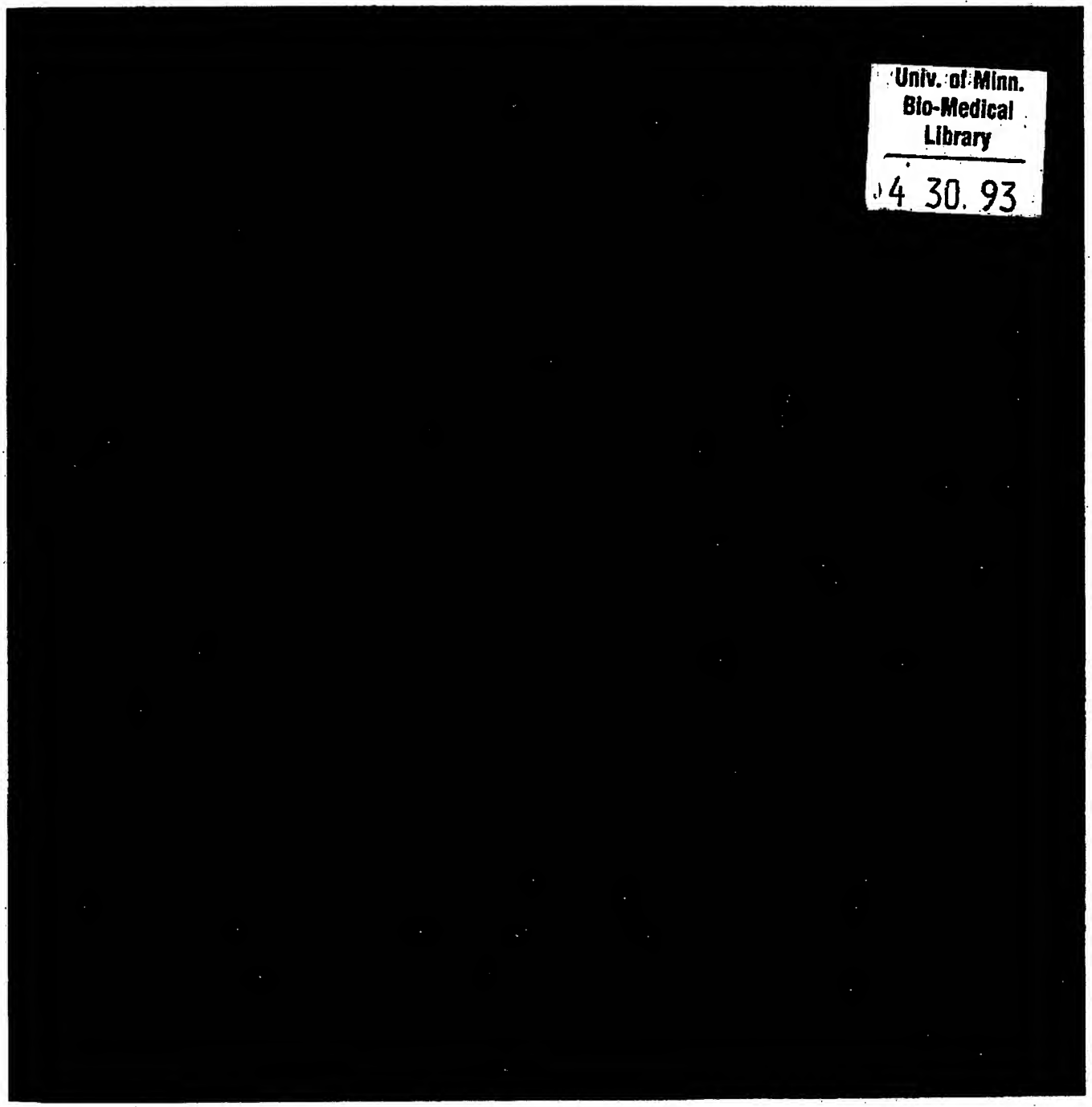

Collagen-induced Arthritis in the BB Rat

Prevention of Disease by Treatment with CTLA-4-Ig

Debbie Barney Knoerzer, Robert W. Karr, Benjamin D. Schwartz, and Laurel J. Mengle-Gaw
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Abstract

Antigen-specific T cell activation requires two independent signalling events, one mediated through T cell receptor engagement by the antigen-presenting cell–expressed peptide/class II major histocompatibility complex, and the second through the cognate interactions of costimulatory molecules expressed on the T cell and antigen-presenting cell. There is evidence from in vitro and in vivo experimental systems suggesting that the CD28/B7 costimulatory pathway is crucial for induction of maximal T cell proliferation and T helper-B cell collaboration for IgG production. This pathway can be blocked by CTLA-4-Ig, a soluble form of CTLA-4 which binds with high avidity to the CD28 ligands, B7-1 and B7-2. Here, we show that CTLA-4-Ig treatment prevents clinical and histological manifestations of disease in a collagen-induced arthritis model of rheumatoid arthritis in the diabetes resistant BB/Wor rat, when therapy is initiated before immunization with bovine type II collagen (BIIC). Anti-BIIC antibody titers are reduced in CTLA-4-Ig–treated rats compared to diseased control animals. Histologically, joints from CTLA-4-Ig–treated animals show no histological abnormalities, in contrast to control antibody-treated animals, which show complete erosion of the articular cartilage and bone. Despite the efficacy of CTLA-4-Ig in preventing clinical and histological signs of arthritis and reducing antibody responses to BIIC, delayed type hypersensitivity responses to collagen 18 d or more after CTLA-4-Ig treatment ends are similar in CTLA-4-Ig–treated and untreated rats, suggesting that the prolonged disease suppression observed does not result from induction of T cell anergy. (*J. Clin. Invest.* 1995. 96:987–993.) Key words: immunosuppression • rheumatoid arthritis • costimulation • anergy

Introduction

Optimal activation of antigen-specific T cells requires the induction of a nonspecific costimulatory signalling pathway, in addition to the primary antigen-specific signal delivered by T cell receptor engagement (1, 2). One such costimulatory accessory molecule is the T cell surface antigen, CD28, whose interaction with its antigen-presenting cell–expressed ligands B7-1 and B7-2 during TCR engagement has been shown to be crucial for maximal T cell signalling (3). B7 ligand binding to CD28 costimulates T cell proliferation and IL-2 transcription (4–6), and monoclonal antibodies directed against both B7 and CD28 specifically block T helper (T_h)-mediated Ig production by B cells in vitro (7). These observations demonstrate the importance of the CD28/B7 costimulus to the functional collaboration between T_h and B cells. The CD28/B7 costimulatory pathway can also be blocked with a chimeric Ig fusion protein of CTLA-4, a CD28 homologue which binds B7-1 and B7-2 with high avidity (8, 9). CTLA-4-Ig binding to B7 has been shown to potently inhibit both T_h proliferation and Ig secretion by B cells in vitro (8).

Antigen stimulation through the TCR in the absence of costimulation can lead to antigen-specific hyporesponsiveness or clonal T cell anergy in vitro (3). For this reason, there has been much interest recently in identifying therapeutic approaches aimed at blocking costimulation, especially in the areas of tissue transplantation and autoimmunity. Because there is no human–rodent species barrier to CTLA-4/B7 interaction, the effects of CD28/B7 pathway blockade using human CTLA-4-Ig constructs have been studied in a variety of rodent-based disease models. In recent in vivo experiments, CTLA-4-Ig treatment has been shown to suppress primary, and to a lesser extent, secondary antibody responses to sheep erythrocytes and keyhole limpet hemocyanin immunogens (10), block xenogeneic pancreatic islet rejection in mice and induce long-term, donor-specific tolerance to the graft (11), increase cardiac allograft survival in rats (12), and block autoantibody production, and prolong survival of mice affected by systemic lupus erythematosis (13). The induction of anergy, rather than prolonged immunosuppression, by CTLA-4-Ig in these systems is variable, and may depend upon the potency of the immunogen, the use of species-matched CTLA-4-Ig, or a variable dependence of certain T cell populations on B7 costimulation.

The diabetes-resistant (DR) BB/Wor rat is a subline of the diabetes-prone BB rat which develops severe, aggressive arthritis upon immunization with heterologous native type II collagen (14). Arthritis develops bilaterally with 100% incidence in the hindpaws, with clinical signs beginning at day +10 after a single collagen injection. Significantly, the DR BB/Wor rat has a major histocompatibility complex (MHC) genetic background relevant to that predisposing to human RA; it shares with RA-susceptible humans a homologous MHC class II–encoded arthritis susceptibility epitope (14). Progression of the disease is associated with infiltration into the periarticular space of mononuclear and multinucleated inflammatory cells and re-

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1. *Abbreviations used in this paper:* BIIC, bovine type II collagen; DR, diabetes resistant; DTH, delayed type hypersensitivity; IFA, incomplete Freund's adjuvant.

sorption of articular cartilage and bone (14). Histologically, DR BB/Wor collagen-induced arthritis resembles human rheumatoid arthritis: multinucleated giant cells, pallisading cells, and pregranulomatous nodules are seen in sections of severely affected joints (Barney Knoerzer, D., B. D. Schwartz, and L. J. Mengle-Gaw, unpublished observations). Antibody mechanisms have been implicated in the pathogenesis of arthritis in this model (14).

In this report, we show that administration of soluble CTLA-4-Ig prevents development of clinical manifestations of collagen-induced arthritis in the DR BB/Wor rat during observation periods up to 62 d. These results demonstrate for the first time that blockade of the CD28/B7 costimulation pathway prevents disease in an in vivo arthritis model.

Methods

Animals. DR BB/Wor rats were obtained from the University of Massachusetts, Worcester, MA, breeding colony. Equal numbers of males and females, aged 45–65 d (~ 150 grams), were used in these studies.

Preparation of CTLA-4-Ig and control Ig. A soluble fusion protein consisting of the extracellular domain of human CTLA-4 and a mouse IgG2a Fc was prepared. The Fc region of mouse IgG2a was generated by reverse transcription followed by PCR amplification using polyA+ RNA isolated from the L243 hybridoma (ATCC #HB 55; American Type Culture Collection, Rockville, MD) and the IgG2a-specific sense primer 5′-GATCGGATCCGAGCCCAGAGGGCCCACAATCAAGCC-3′ and antisense primer 5′-GATCAAGCTTAGATCTTATCATTTACCCGGAGTCCGGGAGAA-3′ (15). The PCR product was digested with BamHI and BglII and cloned into the BamHI site of pMON3360B (16) yielding plasmid pMON24210. DNA corresponding to the signal peptide and extracellular domain of CTLA-4 was generated by PCR amplification using a thymus sDNA library (HL1074a; Clontech, Palo Alto, CA) as template and the CTLA-4–specific sense primer 5′-CCATGGATCCATGGCTTGCCTTGGATTTCAG-3′ and the antisense primer 5′-GATCGGATCCGAAGTCAGAATCTGGGCACGGTTC-3′ (17). The purified BamHI fragment was cloned into the BamHI site of pMON24210 yielding transfection vector pMON24218. Transfection and isolation of a stable cell line expressing 2–5 μg/ml of CTLA-4-Ig were performed as described (16), using Western blot analysis developed with goat anti–mouse Fc to identify the recombinant protein. Recombinant CTLA-4-Ig was purified using protein A-affinity chromatography. An ascites-purified mouse myeloma IgG2a antibody, UPC-10 (Sigma Chemical Co., St. Louis, MO), directed against β1-6 fructosan, was used as a control. Rats were injected intraperitoneally with a 1-mg/kg dose of CTLA-4-Ig or UPC-10 control antibody (1 mg/ml in PBS) at days −1, 0, +2, +4, +6, +8, and +10 (total of seven doses), relative to bovine type II collagen (BIIC) immunization at day 0. Six rats per antibody treatment group were followed through these studies. Data from one of three experiments giving identical results are reported here.

DR BB/Wor rat immunization with bovine II collagen. Bovine IIC was supplied by Dr. Marie Griffiths, University of Utah, Salt Lake City, UT (18). Rats were injected intradermally at the base of the tail with 100 μg BIIC emulsified in incomplete Freund's adjuvant (IFA) (Difco Laboratories Inc., Detroit, MI). The emulsion was made by homogenizing one part native BIIC (4 mg/ml in 0.1 M acetic acid) into one part IFA at 4°C.

Clinical scoring of arthritis in BIIC-immunized DR BB/Wor rats. Clinical severity of arthritis development in the rat paws was scored on a subjective scale ranging from 1–4 for each paw: 1-redness; 2-swelling; 3-digit deformity; 4-ankle deformity (full ankylosis). All clinical score measurements were performed without knowledge of the treatment regimen the rats received.

Determination of serum anticollagen antibody titers. Sera for determination of IgG anti-BIIC antibody titers were obtained by retroorbital bleeding on various days after collagen immunization or from naive age-matched animals. Serum samples were stored at −20°C and were heat inactivated before testing by ELISA. Briefly, microtiter plates (Nunc Immuno Maxisorp, Kirkegard and Perry Laboratories, Inc., Gaithersburg, MD) were coated with 0.1 ml of 0.01 mg/ml native BIIC in phosphate buffer at 4°C overnight. After washing, the wells were blocked with ELISA buffer (1% BSA in PBS). Serum samples, diluted 1:500, 1:2,500, and 1:5,000 in ELISA buffer, were added to wells and incubated 2 h at 25°C. BIIC-bound serum IgG were detected by a horseradish peroxidase–conjugated goat anti–rat IgG (Accurate Chemical and Scientific Corp., Westbury, NY), and plates were developed using ABTS peroxidase substrate (Kirkegard and Perry Laboratories, Inc.) and read at 405 nm on an automated microplate reader (model 3550; Bio-Rad Laboratories, Richmond, CA). Results are expressed as OD units (experimental wells minus baseline OD). A serum with known high levels of anti-BIIC antibodies was used to generate a highly reproducible standard curve as part of each experiment.

Histology. Relevant paws taken from animals killed at the end of the study were skinned and placed in 10% buffered phosphate formalin (Fisher Scientific Co., Pittsburgh, PA) for > 1 wk before being subjected to acid decalcification (19). Decalcified paws were embedded in paraffin, longitudinally sectioned through the center of the tibia–tarsal joint (Histo Techniques, Powell, OH), and stained with hematoxylin and eosin (19).

Delayed type hypersensitivity (DTH) analysis. Lyophilized bovine type II collagen was prepared by the method of Griffiths et al. (18). 2 μg in 0.05 ml was injected intradermally at shaved sites on the back at various days after collagen immunization. Saline buffer injections (0.05 ml) were used as controls. The injection sites were scored and measured at 48 h. Reactive sites were measured in two directions with calipers, and results expressed as mean±SD diameter of induration.

Statistical analysis. A two-factor repeated measures analysis of variance was performed using the PROC MIXED program in SAS (SAS Institute, Inc., Cary, NC) to compare the anti-BIIC antibody levels and DTH responses in the two treatment groups (CTLA-4-Ig or UPC-10) at each day of measurement.

Results

CTLA-4-Ig administration prevents collagen-induced arthritis in the DR BB/Wor rat. The arthritis which develops in the DR BB/Wor rat after a single intradermal injection of heterologous type II collagen shows a rapid clinical and histological progression (14; Barney Knoerzer, D., B. D. Schwartz, and L. J. Mengle-Gaw, unpublished observations). Early clinical signs of hindpaw disease appear at day +10 after BIIC immunization, with redness in one or both hindpaws, progressing to full hindpaw ankylosis by day +16 to day +18 in 100% of immunized rats. Clinical signs of arthritis can also appear in the forepaws of 20–80% of immunized animals, but forepaw disease is generally less severe than hindpaw disease and its development follows hindpaw disease in affected rats (Barney Knoerzer, D., B. D. Schwartz, and L. J. Mengle-Gaw, unpublished observations).

Fig. 1 *A* shows the result of administration of CTLA-4-Ig to six rats, beginning at day −1, with bovine type II collagen immunization on day 0. CTLA-4-Ig treatment was continued 3–4 times weekly until day +10, when all treatment was discontinued. Animals were followed clinically to day +50 in this experiment. At this dose of CTLA-4-Ig, none of the six rats showed any clinical signs of arthritis during the observation period. This outcome compares dramatically to arthritis development in the UPC-10 antibody–treated control group, where, as with saline-treated control animals (data not shown), aggressive hindpaw arthritis began at day +10 and progressed to full

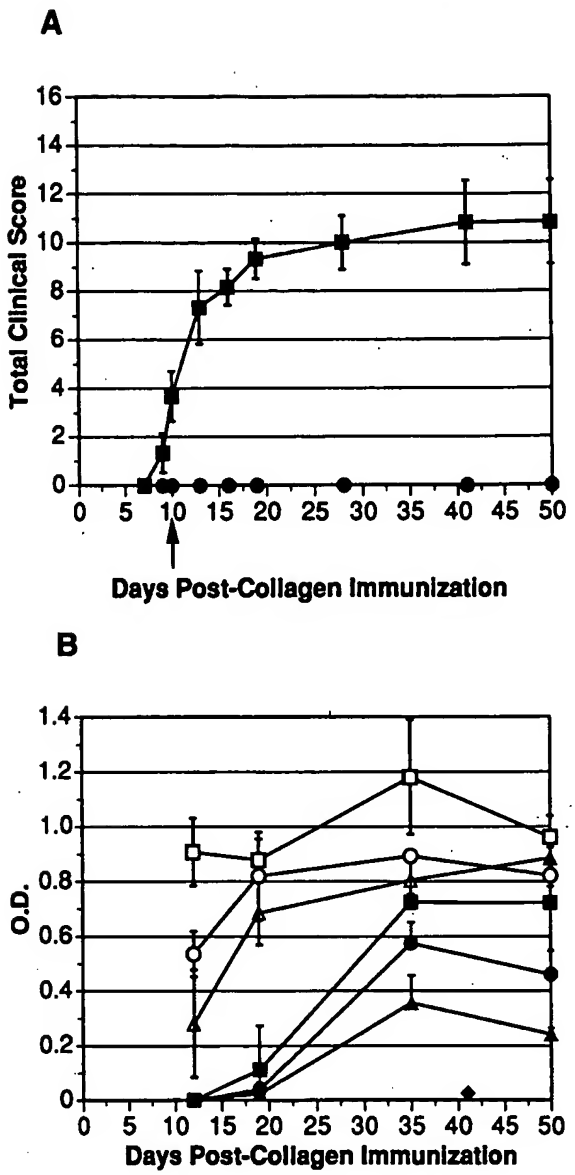

Figure 1. (*A*) Effect of CTLA-4-Ig treatment on the clinical course of arthritis progression in DR BB rats. All rats were immunized with bovine type II collagen at day 0. (— ■ —) Mean clinical score of six control rats treated with UPC-10 antibody; (—●—) Mean clinical score of six rats treated with CTLA-4-Ig. A total of seven doses of either antibody was given between day −1 and day +10. Arrow indicates day of treatment cessation. (*B*) Mean anti-BIIC IgG antibody titers, measured by ELISA at three serum dilutions, of three of the six rats treated with UPC-10 (— □ — 1:500; — ○ — 1:2500; — △ — 1:5000) or CTLA-4-Ig (—■— 1:500; —●— 1:2500; —▲— 1:5000). (— ♦ —) Mean anti-BIIC antibody titer of naive BB littermates.

ankylosis by day +16 (Fig. 1 *A*). Forepaw involvement in the UPC-10–treated animals began at day +19, raising the total clinical score above eight. The data presented are from one of three separate experiments giving identical results. In the other two experiments, four of four and four of four CTLA-4-Ig–treated animals had clinical scores of zero through the 47 and 62-d observation periods, respectively, whereas all of the eight UPC-10–treated rats developed arthritis by day +12 (data not shown).

Humoral response to bovine type II collagen in CTLA-4-Ig–treated rats. Sera from three rats in each of the CTLA-4-Ig and UPC-10–treated groups were analyzed by ELISA for anti-BIIC IgG antibodies after collagen immunization. These results from three serum dilutions at multiple time points are shown in Fig. 1 *B*. Anti-BIIC antibody levels of UPC-10–treated control animals rose rapidly during the first 19 d, in parallel with the development of arthritis in these animals, then persisted at high levels during the remainder of the observation period. Although antibody levels in CTLA-4-Ig–treated rats also rose over time, the antibodies consistently appeared later than in the control-treated rats and the levels were significantly lower at each time point analyzed than in the control-treated rats ($P < 0.05$). Sera from naive age-matched rats have a mean OD of 0.025 at day +41 (Fig. 1 *B*). Anti-BIIC antibody levels were also measured at three serum dilutions in day +12, +19, and +48 samples from three UPC-10–treated rats and three CTLA-4-Ig–treated rats from another experiment and similar results were obtained (data not shown). IgG2a antibodies directed against the CB11 fragment of type II collagen have been identified in the sera of diseased BBN rats (14), and we have previously observed a positive correlation between anti-BIIC titer and disease development in this model (Barney Knoerzer, D., B. D. Schwartz, and L. J. Mengle-Gaw, unpublished observations), suggesting that disease may be mediated at least in part by humoral responses to the heterologous type II collagen used as immunogen. This contention is supported by the observation that mild arthritis can be transferred to naive animals with serum from diseased animals (Anderson, G., D. Barney Knoerzer, B. D. Schwartz, and L. J. Mengle-Gaw, unpublished observations).

Histological analysis of CTLA-4-Ig–treated joints. Hindpaw joints from immunized CTLA-4-Ig–treated rats were examined at day +47 by light microscopy and compared to joints from normal, unimmunized rats, and immunized rats (day +47) receiving UPC-10 control antibody treatment. These sections are compared in Fig. 2. Compared to the normal joint architecture seen in unimmunized animals (Fig. 2 *A*), the joints from BIIC-immunized, UPC-10 control–treated rats show significant pannus formation and complete erosion of cartilage and bone at day +47 (Fig. 2 *B*). By contrast, joints from BIIC-immunized, CTLA-4-Ig–treated rats show no histological abnormalities at day +47 (Fig. 2 *C*).

Cellular response to bovine type II collagen in CTLA-4-Ig–treated rats. Skin delayed type hypersensitivity responses to BIIC were measured in CTLA-4-Ig-treated and control animals (Table I). 4 rats/treatment group were assessed at day +22 and +28 after collagen immunization. The same rats were used for DTH measurement at both day +22 and +28, and were a subset of those whose clinical scores and anti-BIIC antibody titers are shown in Fig. 1. At day +22, the mean DTH score for the CTLA-4-Ig–treated animals was 0.9, with three of the four animals showing no DTH response; this compares with mean DTH scores of 5.2 and 4.7 for the UPC-10–treated and saline-treated control groups, respectively, and represents a significant difference in the ability of CTLA-4-Ig vs UPC-10–treated rats to generate DTH responses to collagen at day +22 ($P < 0.005$). By contrast, the DTH responses at day +28 of

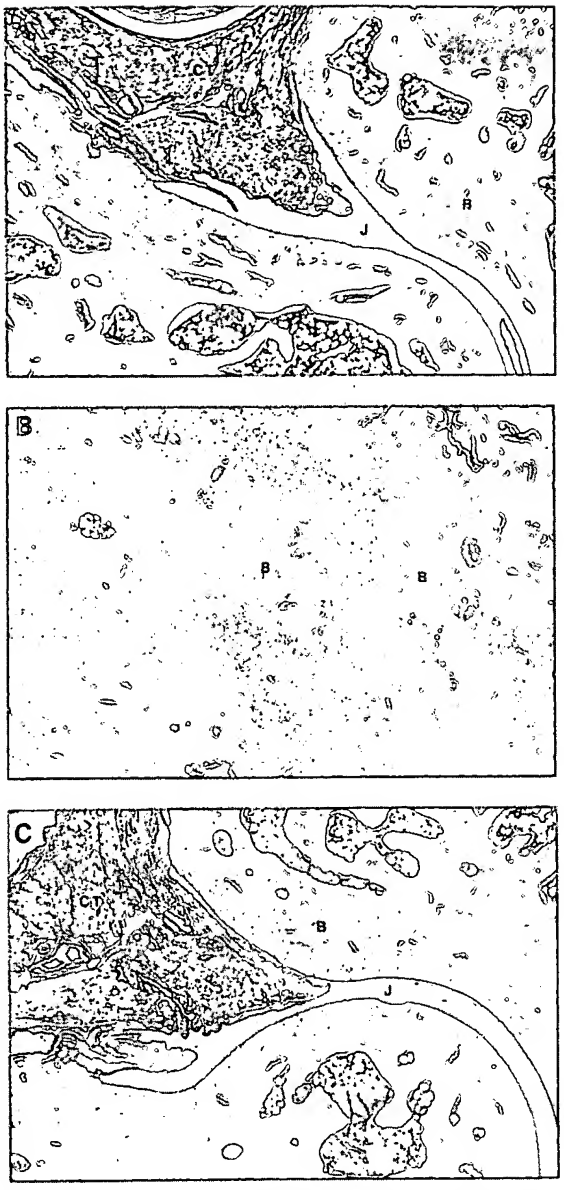

Figure 2. Histological evaluation of the effect of CTLA-4-Ig treatment on arthritis development in the tibia–tarsal joints of BIIC-immunized DR BB rats. Light micrographs were taken of hematoxylin and eosin–stained sections from (*A*) normal, (*B*) UPC-10-treated, arthritic, and (*C*) CTLA-4-Ig–treated DR BB rats 47 d after immunization with bovine CII in IFA. B, bone; J, joint space; CT, connective tissue.

the CTLA-4-Ig–treated animals (mean score = 5.1) are the same as those of control rats receiving either saline (mean score = 4.6) or UPC-10 antibody treatment (mean score = 5.3) (Table I). Therefore, although three of four CTLA-4-Ig–treated animals lack DTH responses to BIIC early after CTLA-4-Ig treatment ends, treated animals regain their ability to generate antigen-specific DTH responses with time; at day +28, CTLA-4-Ig-treated rats show no clinical signs of disease, but do generate normal DTH responses to BIIC. To address the possibility

Table I. Cellular Immunity to Bovine Type II Collagen in DR BB Rats

Treatment Group	Day +22		Day +28	
	No. positive*/ No. tested	Mean diameter‡ of induration±SD	No. positive*/ No. tested	Mean diameter‡ of induration±SD
		mm		*mm*
BIIC + saline	2/2	4.7±0.3	2/2	4.6±0.9
BIIC + CTLA-4-Fc	1/4	0.9±1.9	4/4	5.1±1.3
BIIC + UPC-10	4/4	5.2±0.7	4/4	5.3±1.3
Saline alone	0/2	0.0±0.0	0/2	0.0±0.0

Groups of rats were immunized with BIIC and treated with UPC-10 or CTLA-4-Fc as described in Methods, except the rats in the saline-treated group, who were neither immunized nor treated, and who received saline alone. DTH responses were measured at day +22 and day +28 on the same animals and expressed as the mean±SD diameter of induration (*mm*). * Positive DTH response: mean diameter of induration >3 mm. ‡ Mean±SD calculated using all tested animals in each treatment group.

that the positive DTH responses at day +28 were due to antigen priming with collagen during DTH testing at day +22, additional experiments were done: In two separate experiments, four of four and six of six CTLA-4-Ig–treated rats without arthritis had positive DTH responses to collagen on first testing at day +29 or +36, respectively (data not shown). No CTLA-4-Ig–treated rats developed arthritis following DTH testing.

Collagen rechallenge. In pilot studies, we observed that a CTLA-4-Ig–treated rat without arthritis at day +33 after initial collagen immunization developed robust arthritis after rechallenge with collagen and IFA at day +33. We subsequently conducted a collagen rechallenge experiment with and without the administration of CTLA-4-Ig during the rechallenge, using the six CTLA-4-Ig–treated rats shown in Fig. 1 *A*. At day +50 after the initial collagen immunization and CTLA-4-Ig treatment, each of the CTLA-4-Ig–treated rats had a clinical score of zero. The CTLA-4-Ig–treated rats were divided into two treatment groups in association with collagen rechallenge (BIIC 100 μg in IFA) at day +50: two rats received no additional CTLA-4-Ig treatment, and four rats received seven doses of CTLA-4-Ig during days +47 to +56 (Fig. 3). The untreated

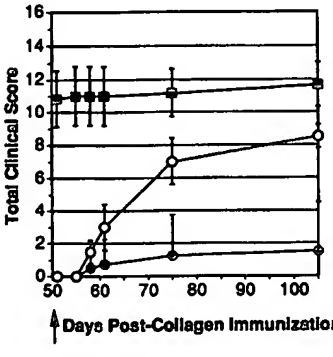

Figure 3. Effect of CTLA-4-Ig treatment of clinical course of arthritis after collagen rechallenge. Rats were rechallenged with collagen at day +50 (*Arrow*). (— o —) Rats rechallenged with collagen without additional CTLA-4-Ig treatment (n = 2), (—●—) rats rechallenged with collagen while receiving CTLA-4-Ig treatment (n = 4), and, for comparison, (— ⊡ —) rats that received UPC-10 control antibody during the initial collagen challenge and developed arthritis (n = 6).

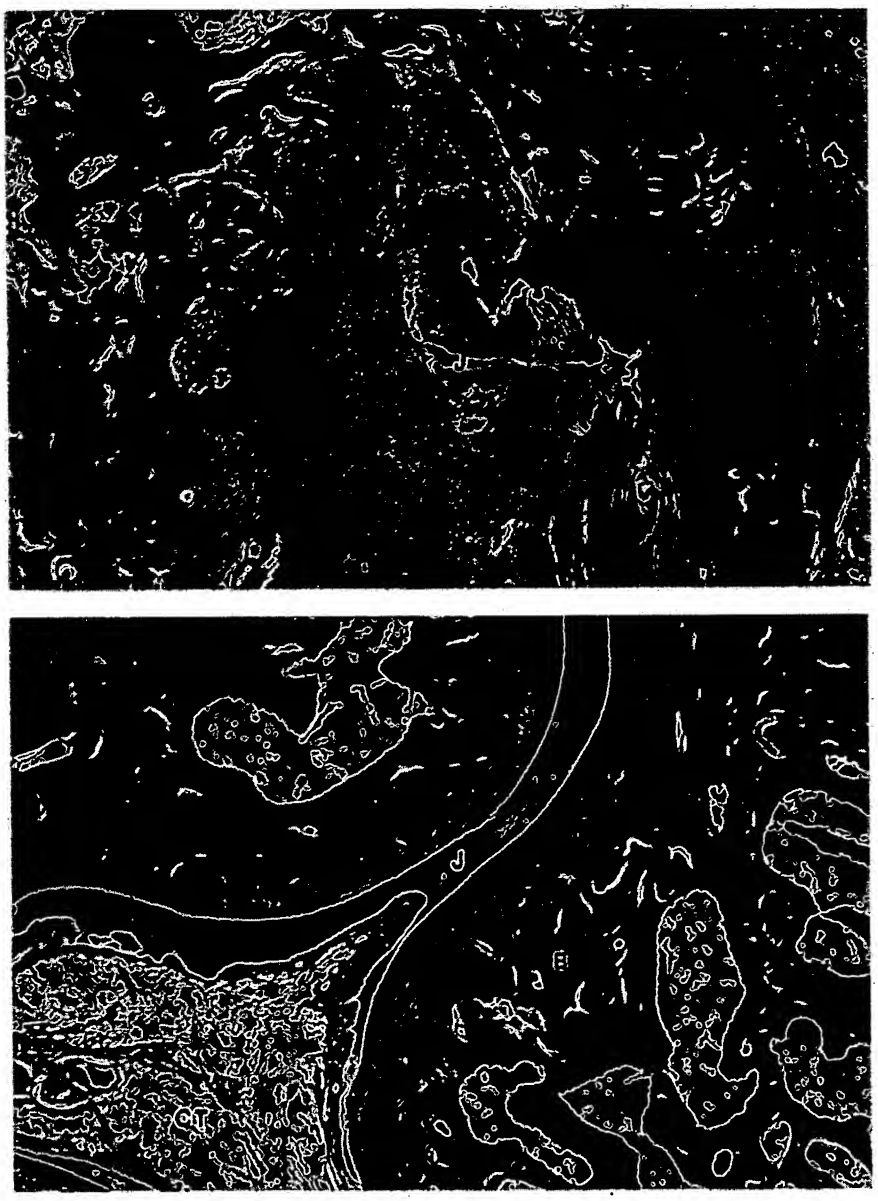

Figure 4. Histological comparison of tibia–tarsal joint sections from BIIC-rechallenged rats. BIIC-immunized, CTLA-4-Ig–treated rats were rechallenged with BIIC at day +50 with concomitant treatment with CTLA-4-Ig, and light micrographs were taken at day +105 of hematoxylin and eosin–stained sections from (*A*) the single rat that developed clinical signs of arthritis (see text), and (*B*) a rechallenged, CTLA-4-Ig–retreated rat that developed no clinical arthritis. B, bone; J, joint space; CT, connective tissue.

rats developed arthritis after collagen rechallenge, with clinical scores of eight and six, respectively, at day +75 and maximum scores of nine and eight during the observation period. The kinetics of arthritis development in these rats were similar to those seen in untreated rats in response to primary collagen immunization (Fig. 1 *A*). Three of the rats treated with CTLA-4-Ig during the rechallenge did not develop arthritis (clinical scores, zero) through the observation period to day +105. One of the CTLA-4-Ig–treated rats developed arthritis by day +61 (clinical score, three), with a maximum score of six at day +105. Anti-BIIC antibody levels remained comparably elevated in the CTLA-4-Ig–treated rat that developed arthritis and in those without arthritis (data not shown). At day +105, histopathology of joints from rats that did and did not develop arthritis after collagen rechallenge was compared (Fig. 4). A hindpaw tibia–tarsal joint from the CTLA-4-Ig–treated rat that developed moderate arthritis following rechallenge shows significant pannus formation and erosion of cartilage and bone (Fig. 4 *A*). By comparison, joints from rechallenged CTLA-4-Ig–treated rats without arthritis showed no histological abnormalities (Fig. 4 *B*).

Discussion

Collagen-induced arthritis in the DR BB/Wor rat is a severe, aggressive disease that affects 100% of immunized animals and proceeds from synovial hypertrophy to pannus formation and articular cartilage and bone destruction within 20 d after heterol-

ogous type II collagen injection (14; Barney Knoerzer, D., B. D. Schwartz, and L. J. Mengle-Gaw, unpublished observations). The histological changes observed during disease progression resemble those seen in rheumatoid arthritis, albeit on a much accelerated temporal scale (Barney Knoerzer, D., and L. Mengle-Gaw, manuscript in preparation); thus collagen-induced arthritis in the DR BB/Wor rat may serve as a useful in vivo disease model for the testing of potential therapeutics relevant to RA. We have shown that treatment with soluble CTLA-4-Ig prevents clinical development of arthritis in the DR BB/Wor rat, when treatment is initiated before collagen immunization. Furthermore, three of four animals receiving a second course of CTLA-4-Ig treatment during rechallenge with collagen were protected from developing clinical signs of arthritis.

Treatment with CTLA-4-Ig between day −1 and day +10 prevented development of clinical manifestations of arthritis (redness, swelling, and digit and ankle deformity) during prolonged observation periods in this model in which 100% of control-treated rats develop persistent arthritis beginning by day +10. Throughout the 50 or more postimmunization days of the observation periods for these experiments, CTLA-4-Ig–treated rats showed no histological abnormalities, with no evidence of the cartilage and bone resorption which completely obliterates the joint architecture of control joints by day +19 (Barney Knoerzer, D., and L. Mengle-Gaw, manuscript in preparation).

Anticollagen antibodies have been implicated in the pathogenesis of disease in the DR BB/Wor rat, as in other rodent collagen arthritis models (20). Arthritic DR BB/Wor rats have high titers of IgG2a anticollagen antibodies (14), and we have shown that passive transfer of serum from arthritic DR BB/Wor rats to naive rats is sufficient to evoke the cartilage and bone erosion associated with full-blown disease (Anderson, G., D. Barney Knoerzer, B. D. Schwartz, and L. J. Mengle-Gaw, unpublished data). By analogy to other rodent collagen-induced arthritis models, these antibodies, initially present in the circulation, may cross-react with type II collagen present in the cartilage of the joint, forming localized antigen–antibody complexes (21, 22). Activated complement components presumably induce the migration of inflammatory cells into the joint. Inflammatory cells are observed at the synovial margins of the joints of BIIC-immunized DR BB/Wor rats by day +9 after collagen injection (Barney Knoerzer, D., and L. Mengle-Gaw, manuscript in preparation). It is unclear whether humoral responses to collagen are involved in the etiology of human RA: Only a small proportion of RA patients have detectable levels of anticollagen antibodies in their sera. However, the majority of RA patients test positive for anticollagen antibodies produced by synovial tissue–derived B cells (23), suggesting that anticollagen autoimmunity may indeed play a role in RA pathogenesis.

Although data from other studies indicate a role for anti-BIIC antibodies in the pathogenesis of collagen-induced arthritis in the BB rat, CTLA-4-Ig–treated rats produced anti-BIIC antibodies but did not develop arthritis. However, the anti-BIIC antibody levels in CTLA-4-Ig–treated rats were consistently lower than in control-treated rats. In other model systems, inhibition or absence of CD28 costimulation has been shown to decrease Th-mediated antibody responses (10, 24) and T cell proliferation (25). The prevention of arthritis by CTLA-4-Ig in our studies suggests that T cell proliferation may be more susceptible to inhibition by CTLA-4-Ig than antibody production and that both T cell proliferation and anti-BIIC antibodies are required to produce arthritis.

In spite of the absence of arthritis, CTLA-4-Ig–treated rats display positive delayed type hypersensitivity responses to bovine IIC at day +28, comparable to those observed in immunized, control-treated animals. This result clearly indicates that CTLA-4-Ig treatment has not caused antigen-specific T cell hyporesponsiveness, or anergy, in these rats. The inability of CTLA-4-Ig–treated rats to generate BIIC-specific DTH responses early in the experimental course may reflect the presence of residual serum CTLA-4-Ig, a hypothesis supported by results of in vivo studies showing slow serum clearance rates for CTLA-4-Ig (10, 26). Prolonged immunosuppression without anergy has been reported in other in vivo experimental systems, where CTLA-4-Ig has been used to block T-dependent antibody responses, and has been suggested to result from variable dependence of certain T cell populations on B7 costimulation (10). Our data indicate that CTLA-4-Ig treatment prevents collagen-induced arthritis and protects rats rechallenged with collagen from developing arthritis, presumably by blocking activation of arthritis-inducing T cells, but does not prevent generation of cells that could participate in a DTH response to collagen. The immunoregulatory network that prevents the in vivo activation of these cells remains to be defined.

The data reported herein suggest that blockade of the CD28/B7 costimulatory pathway may be efficacious for the treatment of human rheumatoid arthritis.

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Prevention of Systemic Lupus Erythematosus in Autoimmune BXSB Mice by a Transgene Encoding I-E α Chain

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Summary

Males from the BXSB murine strain (H-2^b) spontaneously develop an autoimmune syndrome with features of systemic lupus erythematosus (SLE), which results in part from the action of a mutant gene (*Yaa*) located on the Y chromosome. Like other H-2^b mice, the BXSB strain does not express the class II major histocompatibility complex antigen, I-E. Here we report that the expression of I-E ($E\alpha^d E\beta^b$) in BXSB males bearing an $E\alpha^d$ transgene prevents hypergammaglobulinemia, autoantibody production, and subsequent autoimmune glomerulonephritis. These transgenic mice bear on the majority of their B cells not only I-E molecules, but also an I-E α chain–derived peptide presented by a higher number of I-A^b molecules, as recognized by the Y-Ae monoclonal antibody. The I-E^+ B cells appear less activated in vivo than the I-E^- B cells, a minor population. This limited activation of the I-E^+ B cells does not reflect a functional deficiency of this cell population, since it can be stimulated to IgM production in vitro by lipopolysaccharides at an even higher level than the I-E^- B cell population. The development of the autoimmune syndrome in the transgenic and nontransgenic bone marrow chimeric mice argues against the possibility that the induction of regulatory T cells or clonal deletion of potential autoreactive T cells as a result of I-E expression is a mechanism of the protection conferred by the $E\alpha^d$ transgene. We propose a novel mechanism by which the $E\alpha^d$ transgene protects BXSB mice against SLE: overexpression of I-E α chains results in the generation of excessive amounts of a peptide displaying a high affinity to the I-A^b molecule, thereby competing with pathogenic autoantigen–derived peptides for presentation by B lymphocytes and preventing their excessive stimulation.

The BXSB mouse strain spontaneously develops a progressive and lethal autoimmune disease, similar to human SLE, which affects male animals much earlier than females (1, 2). Cell transfer and Y chromosome transfer studies have clearly demonstrated that the Y chromosome-linked autoimmune acceleration (*Yaa*) gene present in the Y chromosome of the BXSB strain is responsible, in mice predisposed to autoimmune diseases, for the accelerated autoimmune abnormalities and immunopathological lesions (3–6). In addition, it has been demonstrated that the MHC genes play a critical role in the development of SLE-like autoimmune syndrome in BXSB mice (7) and in their F_1 hybrids with NZB mice (Merino, R., M. Iwamoto, and S. Izui, manuscript in preparation): the H-2^b haplotype appears to be associated with the development of the autoimmune disease, whereas the H-2^d haplotype protects against this disease. Since BXSB and (NZB × BXSB)F_1 mice bearing the H-2^b haplotype do not express one of the class II MHC antigens, I-E (because of the deletion of the promoter region of the $E\alpha$ gene [8]), the inhibitory effect of the H-2^d haplotype may in part be related to the expression of the I-E molecule. In fact, it has been shown that I-E molecules could exhibit a suppressive activity on immune responses (9), and more recently the expression of I-E molecules through an I-E α chain transgene was found to result in the prevention of autoimmune diabetes in NOD (I-E^-) mice (10–13). In the present study, we found that the expression of a transgene encoding the I-E α chain, $E\alpha^d$, is highly protective against the development of the lupuslike autoimmune disease in BXSB mice, and defined the possible protective mechanism(s) conferred by the expression of this transgene.

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Materials and Methods

Generation of BXSB Eα^d Transgenic and BXSB.Igh^a Congenic Mice. BXSB mice were purchased from The Jackson Laboratories (Bar Harbor, ME). A 14-kb SacII/XhoI fragment containing the entire Eα^d gene sequence (14) was microinjected into fertilized eggs of BXSB mice, as described (15). Mice were screened for the transgene by the surface staining of PBMC using an anti-I-E mAb, Y-17 (16), as described below.

BXSB.Igh^a congenic mice were established by transfer of the Igh^a gene complex of the B.C20/Icr strain as follows: BXSB females were mated with B.C20/Icr males, male offsprings which carry the Igh^a allotype, as determined by ELISA (described below), were then backcrossed with BXSB females for 11 generations. Then, females carrying the Igh^a allotype were mated with BXSB males to introduce the *Yaa* gene. The BXSB.Igh^a novel strain was obtained by mating female and male BXSB Igh$^{a/b}$ heterozygotes at the 12th backcross generation.

Southern Blot Analysis. 5 μg of high molecular weight genomic DNA prepared from tails of BXSB mice were digested with SacI, electrophoresed on a 1.0% agarose gel, and transferred to a nylon membrane (GeneScreen Plus; Du Pont Co., Boston, MA) as described (17). Hybridization was carried out under high stringency with a ^{32}P-labeled 2.6-kb SalI fragment containing the first exon of the Eα^d gene (18), which recognizes two 1.9 and 2.9-kb fragments of the injected Eα^d gene and a 4.2-kb fragment of the endogenous defective Eα^b gene in SacI-digested DNA (see Fig. 1 *A*).

Northern Blot Analysis. Total RNA was extracted, using the guanidine isothiocyanate/CsCl method (19), from spleen, thymus, lymph nodes, liver, kidneys, lungs, and brain of BXSB mice. RNA (30 μg) were electrophoresed on a 1% formaldehyde-containing agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-labeled 3.5-kb SalI fragment containing exons 2, 3, and 4 of the Eα^d gene (18) or ^{32}P-labeled cDNA corresponding to β-actin.

Cytofluorometric Analysis. The expression of I-E and/or I-A molecules in peripheral blood and spleen cells was analyzed by staining first with FITC-conjugated anti-mouse μ chain mAb (LO-MM-9) (20), and then incubating with biotinylated anti-I-E (Y-17) and/or anti-I-A^b (Y-3P) mAb (21), followed by PE-conjugated avidin (Caltag Laboratories, San Francisco, CA) and analyzed with a FACScan® (Becton Dickinson & Co., Mountain View, CA). The expression of the I-A^b-Eα peptide complexes, recognized by the Y-Ae mAb (22, 23), was determined by two different staining procedures. First, spleen cells were first stained with FITC-conjugated anti-mouse μ chain mAb (LO-MM-9), and then incubated with biotinylated Y-Ae mAb, followed by PE-conjugated avidin. Second, spleen cells were first stained with FITC-labeled anti-I-E (Y-17) mAb, then with PE-conjugated goat anti-mouse μ chain Abs (Caltag Laboratories), and incubated with biotinylated Y-Ae mAb, followed by Streptavidin-RED670™ (Gibco BRL, Gaithersburg, MD). The expression of Vβ segments of TCR in Thy-1$^+$ lymph node cells was analyzed as described previously (7). Monocytes in peripheral blood were enumerated using anti-Mac-1 mAb (M1/70) (24) followed by FITC-conjugated goat anti-rat IgG Abs. Surface IgM^a and IgM^b positive cells in peripheral blood from bone marrow chimeric mice were stained with biotinylated mAb, RS-3.1 (anti-IgM^a) (25), and MB86 (anti-IgM^b) (26), followed by PE-conjugated avidin.

Histopathology. Samples of all major organs were obtained at autopsy, and histological sections were stained with either the periodic acid-Schiff reagent or with hematoxylin eosin. Glomerulonephritis was scored on a 0–4 scale based on the intensity and extent of histopathological changes, as described previously (5).

Serological Assays. Serum levels of total IgG, IgG Abs against single-stranded DNA or human IgG (HGG)[1] and the allotype of anti-DNA Abs were determined by ELISA as described (6, 27, 28). Anti-DNA or anti-HGG activities in sera are expressed in titration units as described previously (6, 27). Serum levels of gp70–anti-gp70 immune complexes (gp70 IC) were quantified by an ELISA combined with the precipitation of the serum with polyethylene glycol (average molecular weight 6,000), as described (6, 7). Results are expressed as μg/ml of gp70 complexed with anti-gp70 Abs by referring to a standard curve obtained from NZB sera with known amounts of gp70. Serum levels of Igh^a and Igh^b allotypes were determined by a previously described ELISA (28).

Isolation of I-E$^+$IgM$^+$ and I-E$^-$IgM$^+$ B Cells, and Cell Culture. Spleen cells, depleted of Thy-1$^+$ cells by treatment with anti-Thy-1.2 (AT83) mAb (29) and rabbit complement from transgenic BXSB mice were stained for surface IgM and I-E molecules as described above. Then, I-E$^+$IgM$^+$ or I-E$^-$IgM$^+$ B cells were purified by a FACStar® (Becton Dickinson & Co.) For the spontaneous IgM and IgG secretion, 10^5 I-E$^+$IgM$^+$ or I-E$^-$IgM$^+$ B cells were incubated in 200 μl DME supplemented with additional amino acids, 10 mM Hepes, 5×10^{-5} M 2-ME, and 10% FCS for 24 h at 37°C in a humidified incubator containing 5% CO_2 in air. For the LPS-induced IgM production, 3×10^4 I-E$^+$IgM$^+$ or I-E$^-$IgM$^+$ B cells in 200 μl culture were stimulated with 50 μg/ml of *Escherichia coli* LPS (Difco Laboratories, Inc., Detroit, Michigan) for 5 d. IgM and IgG concentrations in supernatants were determined by ELISA (6, 7).

Preparation of Bone Marrow Chimeras. 2-mo-old BXSB female recipients were irradiated at 850 rad and reconstituted with bone marrow cells (BMC) from 3–4-mo-old BXSB mice. A mixture of 10^7 viable BMC were intravenously injected into irradiated recipients as detailed in Table 1.

Statistical Analysis. Statistical analysis was performed with the Wilcoxon two-samples test. Probability values >5% were considered insignificant.

Results and Discussion

To explore a possible protective effect of the I-E molecule on the development of SLE in autoimmune BXSB male mice, we introduced the Eα^d gene into this strain, and nine founder transgenic BXSB mice were generated. A line from one of these founder mice, BXSB-E-1, which contains approximately 50 copies of the Eα^d transgene (Fig. 1 *A*), was established and analyzed in more detail. The Eα transcript was strongly expressed in thymus, spleen, and lymph nodes, and at a low level in lungs, liver, and kidneys (Fig. 1 *B*), in agreement with previous results in I-Eα^d transgenic C57BL/6 (B6.Eα^d) mice (14). Comparative measurements

[1] *Abbreviations used in this paper:* BMC, bone marrow cells; B6.Eα^d, I-Eα^d transgenic C57BL/6; gp70 IC, gp70-anti-gp70 immune complexes; HGG, human IgG.

Table 1. *BMC Chimeric Mice*

Group	BMC donor	Recipient
I*	BXSB.Igh^a male (Igh^a) + BXSB-E-1 male (Igh^b)	BXSB female
II	BXSB.Igh^a male (Igh^a) + BXSB male (Igh^b)	BXSB-E-1 female
III	BXSB.Igh^a male (Igh^a) + BXSB male (Igh^b)	BXSB female
IV	BXSB.Igh^a female (Igh^a) + BXSB-E-1 female (Igh^b)	BXSB female

* 2 mo after the reconstitution, the chimerism was checked by measuring serum levels of Ig allotype and by enumeration of IgM^a and IgM^b allotype-positive circulating B cells. These analyses showed that both populations of donor B cells were equally repopulated in recipient mice (data not shown).

of $E\alpha$ mRNA and β-actin mRNA levels, as determined by densitometric analysis on Northern blots, showed an ~20-fold-greater expression of $E\alpha$ mRNA in the spleen from the BXSB-E-1 mice than in BXSB.H-2$^{b/d}$ heterozygous mice.

Expression of the $E\alpha$ gene product was examined by the surface staining of spleen cells using an anti-IE mAb, Y-17, which recognizes combinations of $E\alpha$ and $E\beta$ molecules of various haplotypes, including $E\alpha^d E\beta^b$ (16). Spleen cells from the BXSB-E-1 mice expressed the I-E molecule exclusively on surface IgM$^+$ B cells at levels similar to those found on the spleen B cells of CBA/J (H-2^k, I-E$^+$) mice (Fig. 1 *C*). The transgenic mice contained, however, a significant percentage of I-E$^-$IgM$^+$ B cells (about 15–20% of splenic B cells), which expressed the I-A^b molecule at a density as high as that found on I-E$^+$IgM$^+$ B cells (data not shown). Such a disparate expression of I-E antigens among B cells has been previously noted in other $E\alpha^d$ transgenic mice generated using transgenes containing only a short 5′ flanking sequence of the $E\alpha^d$ gene (30). However, our $E\alpha^d$ transgene construct contains about 4 kb of 5′ flanking sequence, which is enough to allow an appropriate expression of $E\alpha^d$ mRNA and I-E α chain. In the spleens of B6.Eα^d transgenic mice generated using the same construct (14), no significant numbers of I-E$^-$IgM$^+$ B cells were found (Fig. 1 *C*). The presence of I-E$^-$IgM$^+$ B cells may thus be a peculiarity of the BXSB-E-1 mice or of mice with a lupus background. It is also possible that the I-E$^-$ and I-E$^+$ populations may represent two different subsets of B cells at different stages of activation or differentiation. In the transgenic mice, the I-E molecule was also present on a very small fraction of unstimulated monocytes, but on most IFN-γ-stimulated monocytes, as is also the case for normal I-E$^+$ mice (data not shown). Immunofluorescence staining with the Y-17 anti-I-E mAb of thymus cryosections of these transgenic mice showed typical confluent staining of the medulla and reticular staining of the cortex (data not shown).

Expression of the I-E antigen in these transgenic mice was accompanied by a decrease in the Vβ5$^+$ and Vβ11$^+$ T cells, which potentially contain anti-I-E autoreactive T cells. Vβ5$^+$ and Vβ11$^+$ represented 1.48 and 2.91% of Thy-1$^+$ cells from the BXSB-E-1 lymph nodes, as compared with 7.48 and 5.14%, respectively, in the BXSB nontransgenic I-E$^-$ littermates. In contrast, no differences were found in the percentages of Vβ6$^+$ and Vβ8.2$^+$ cells between the transgenic mice (10.55 and 12.45%, respectively) and their nontransgenic littermates (8.04 and 12.12%). The extent of this depletion of Vβ5$^+$ and Vβ11$^+$ T cells, presumably due to I-E expression in the transgenic mice, was comparable with that observed in I-E$^+$ BXSB H-2$^{b/d}$ heterozygous mice (7). The spleen cells of the BXSB-E-1 mice induced a significant proliferation of I-E$^-$ BXSB lymphocytes in MLC (data not shown).

The lupuslike autoimmune syndrome developing in the male nontransgenic I-E$^-$ littermates was dramatically prevented in the male BXSB-E-1 transgenic mice. Whereas 50% of the nontransgenic I-E male littermates had died of glomerulonephritis within the first 8 mo, with no survivors after 1 yr, none of the transgenic mice died within the first year (Fig. 2 *A*). Kidney histology at 8 mo showed in I-E$^-$ male mice a severe glomerulonephritis with increased mesangial and glomerular cellularity, obliteration of glomerular architecture, and tubular cast formation. In contrast, transgenic males exhibited minimal glomerular changes (Fig. 3). In correlation with the renal lesions, serum levels of total IgG, nephritogenic gp70 IC (6, 7, 31, 32) and IgG anti-DNA autoantibodies in the male transgenic mice at 6 mo were markedly lower than those of their male nontransgenic littermates ($p < 0.001$), and almost comparable with those of female BXSB mice (Fig. 2, *B–D*). Blood monocytosis, a unique abnormality characteristic of conventional BXSB male mice (33) was, however, unaltered in transgenic mice (percent Mac-1$^+$ PBMC at 8 mo of age [mean of 5 mice ± 1 SD]: transgenic males, 20.6 ± 2.6%; transgenic females, 6.6 ± 0.9%; nontransgenic males, 19.5 ± 5.8%; nontransgenic females, 6.2 ± 1.2%). This indicates that monocytosis in BXSB males is neither a cause nor a consequence of the autoimmune syndrome, but rather is somehow related to the nature of the *Yaa* mutation. This also suggests that the expression of the I-E α chain transgene prevents the development of autoimmune responses, rather than the acceleration effect mediated by the *Yaa* gene in BXSB mice. This is further supported by our recent observation that the lupuslike autoimmune syndrome developing in (NZB × BXSB-E-1)F$_1$ female mice in the absence of the *Yaa* gene is similarly inhibited by the presence of the $E\alpha^d$ transgene (Iwamoto, M., R. Merino, and S. Izui, unpublished observation).

The present results demonstrate that the expression of the I-E α chain transgene prevents the autoimmune syndrome

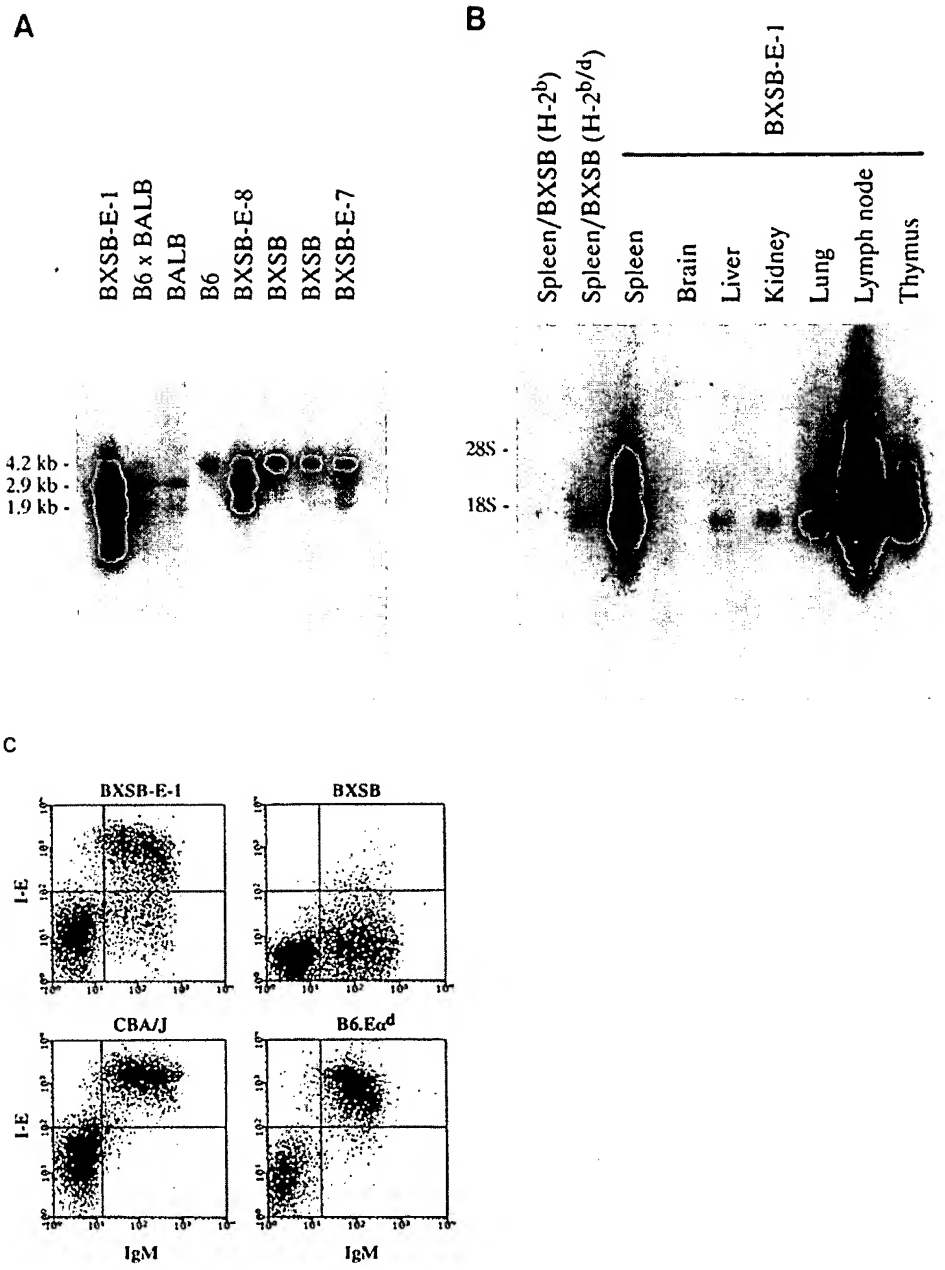

Figure 1. (*A*) Southern blot analysis of the *Eα* gene in transgenic (*BXSB-E-1*, *BXSB-E-7*, *BXSB-E-8*) or nontransgenic BXSB, C57BL/6 (*B6*) (*H-2*^{b}), BALB/c (*H-2*^{d}) and their F_1 hybrids (*B6 × BALB*) mice. The host BXSB mice carry a deletion in this region, as other H-2^b mice (8), and their SacI-digested DNA yielded a 4.2-kb band, larger than those (1.9 and 2.9 kb) obtained from BALB/c DNA. DNA from transgenic mice (*BXSB-E-1*, *BXSB-E-7*, *BXSB-E-8*) gave, in addition, the two 1.9- and 2.9-kb bands characteristic of the *Eα*^{d} gene, indicating that these mice contain the injected BALB/c *Eα*^{d} gene. (*B*) Northern blot analysis of *Eα* mRNA from various tissues of the BXSB-E-1 mice. As controls, RNA from I-E$^+$ BXSB.H-2$^{b/d}$ (7) and I-E$^-$ BXSB (*H-2*^{b}) spleens were also analyzed. Positions of 18S and 28S ribosomal RNA are indicated. (*C*) Expression of the I-E (*Eα*^{d}*Eβ*^{b}) antigen on spleen cells of the BXSB-E-1 mice. Spleen cells from 2-mo-old male transgenic and nontransgenic BXSB, CBA/J (*I-E*$^+$) and B6.*Eα*^{d} transgenic (14) mice was first stained with FITC-conjugated anti-mouse *μ* chain mAb (LO-MM-9), and then incubated with biotinylated anti-I-E (Y-17) mAb, followed by PE-conjugated avidin.

in BXSB mice. This is reminiscent of the prevention of spontaneous autoimmune diabetes in NOD (I-E$^-$) mice by the expression of the I-E antigen through an I-E *α* chain transgene (10–13). However, NOD mice (H-2^{g7}) have a unique I-A^{NOD}, made of an I-A*α*^{d} chain and of a distinct type of I-A *β* chain (34). The mechanisms by which I-E expression protects NOD mice are still unclear, and protection is also afforded by the expression of I-A^k (35, 36). It has been speculated that expression of another class II MHC molecule in the cells of NOD mice prevents the peculiar self-antigen–presenting property of I-A^{NOD}. In contrast, the I-A *α* and *β* chains of BXSB mice are apparently conventional H-2^b class II MHC molecules. Expression of I-E molecules at the level provided by the H-2$^{b/d}$ heterozygous state does not protect BXSB mice from their autoimmune syndrome (7), despite the fact that it is accompanied by a decrease in V*β*5$^+$ and V*β*11$^+$ T cells comparable with that observed in the BXSB-E-1 mice. This suggests that lack of the autoimmune syndrome in the BXSB-E-1 mice may be related to an unusually high level of I-E *α* chains, as suggested by the very high levels of corresponding mRNA detected (probably related to the large copy number of *Eα*^{d} transgenes carried by these

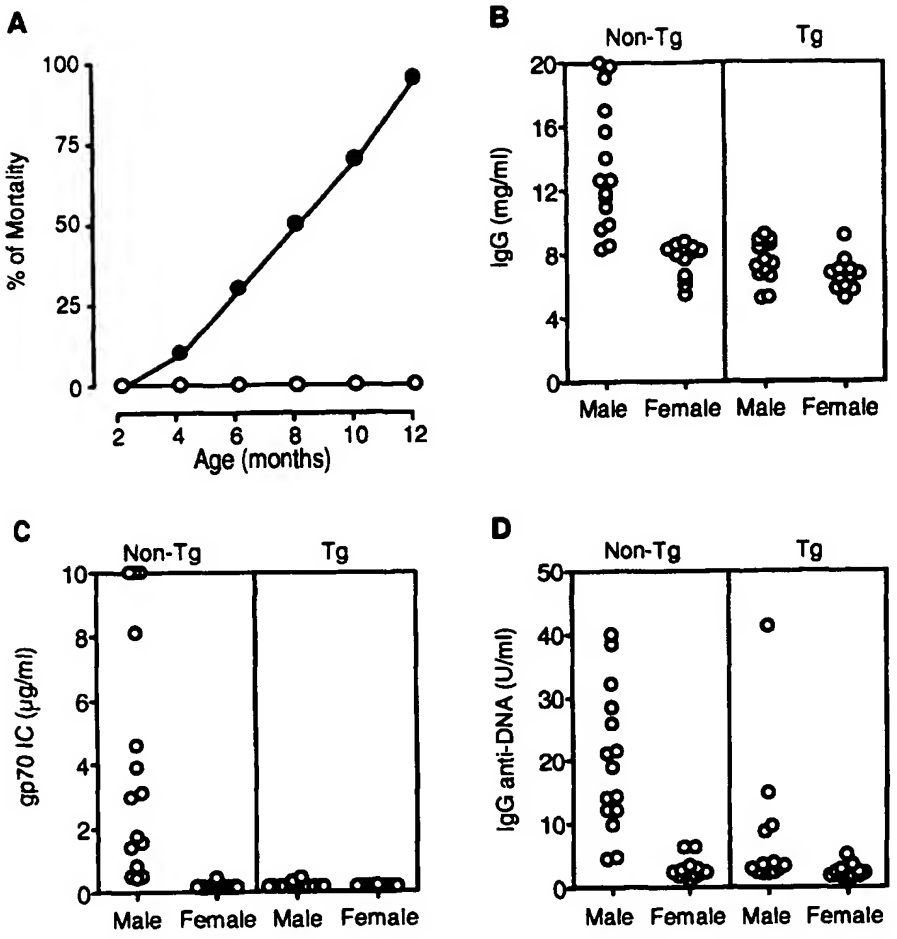

Figure 2. (*A*) Cumulative mortality with glomerulonephritis in male BXSB transgenic (○) and nontransgenic (●) littermates (15 animals from each group). (*B–D*) Serum levels of total IgG, gp70 IC, and IgG anti-DNA autoantibodies in 6-mo-old BXSB transgenic and nontransgenic male and female mice.

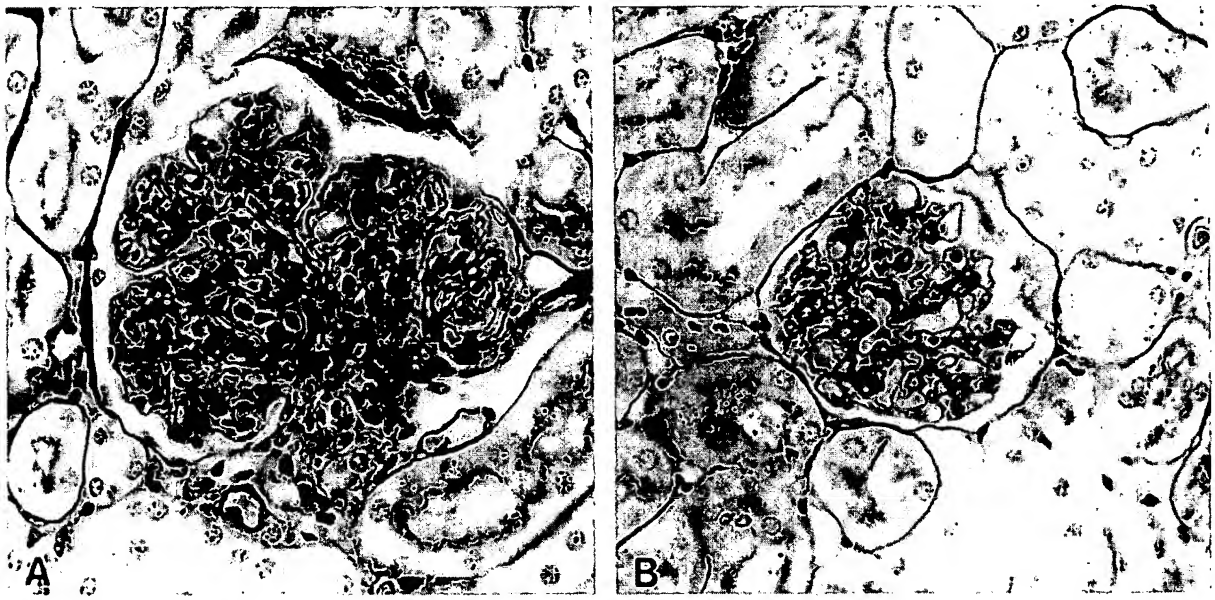

Figure 3. (*A*) Representative histological appearance of glomerular lesions of kidneys from 8-mo-old BXSB nontransgenic male littermates showing increased glomerular cellularities and obliteration of the glomerular architecture. (*B*) Representative histological appearance of glomeruli from 8-mo-old BXSB transgenic males exhibiting minimal glomerular changes. Note a marked difference in the size of glomeruli between BXSB transgenic and nontransgenic littermates. The tissues were stained with periodic acid-Schiff reagent (×200).

mice). Preliminary findings indicate that the second transgenic line, BXSB-E-2, which also carries a high copy number of $E\alpha^d$ transgenes and expresses $E\alpha$ mRNA at a level similar to the BXSB-E-1 mice, fails to develop the autoimmune syndrome. An immune deficiency syndrome has been observed in mice bearing a high copy number (>50) of the $A\beta^k$ transgene (37). It should be emphasized here that the BXSB-E-1 mice did not show any feature of immune deficiency, had an IgG level in male transgenic mice comparable with that found in nontransgenic BXSB female mice (Fig. 2 *B*), and displayed a normal immune response against the T cell–dependent antigen HGG (serum IgG anti-HGG levels 14 d after aggregated HGG injection: BXSB-E-1, 528 ± 154 U/ml; BXSB, 555 ± 215 U/ml; IgG anti-HGG levels before immunization were <5 U/ml).

Overexpression of I-E α chain in these transgenic mice appears to lead to an increased formation of peptides derived from this chain which are presented in the groove of I-A^b molecules. In mice bearing I-A^b and I-E molecules, it has been shown that one of the major self-peptides presented by the I-A^b molecules is derived from the I-E α chain. It appears that the I-A^b–I-Eα peptide complexes are recognized by the Y-*Ae* mAb (22, 23). When this mAb was used to stain spleen cells of male mice derived from a cross between the BXSB-E-1 and BXSB.H-2^d mice, surface IgM^+ B cells of mice bearing the $E\alpha^d$ transgene expressed a higher density of the Y-*Ae* epitope than similar cells from nontransgenic littermates. This was not due to a difference in density of the I-A^b molecule on these cells, since B cells from both types of mice expressed identical I-A^b density (Fig. 4). The likeliest interpretation of this observation is that on splenic B cells of mice bearing the transgene, an increased fraction of the I-A^b molecules contain in their groove the I-E α chain–derived peptide.

To explore whether an increased presentation of I-E α chain peptides by the I-A^b molecule might prevent excessive activation of the B cells bearing these peptides, and thus explain the lack of autoimmune disease in the BXSB-E-1 mice, splenic $I\text{-}E^+IgM^+$ and $I\text{-}E^-IgM^+$ B cells isolated from 1 yr-old BXSB-E-1 male mice were fractionated and compared for their spontaneous Ig production. As shown in Fig. 5 *A*, $I\text{-}E^-$ B cells secreted four to eight times more IgM and IgG than $I\text{-}E^+$ B cells during a 24-h culture, suggesting that $I\text{-}E^-$ B cells were selectively activated in vivo in the BXSB-E-1 mice. The smaller spontaneous Ig production of $I\text{-}E^+$ B cells was not due to a general functional deficiency of these cells, since after stimulation with bacterial LPS, they were capable of producing IgM Abs at an even higher level than $I\text{-}E^-$ B cells (Fig. 5 *B*). The relatively lower stimulation of $I\text{-}E^-$ B cells in vitro by LPS is consistent with the notion that these latter cells are selectively activated in vivo, as is the case for B cells derived from diseased lupus-prone mice (38).

It may be that a mechanism operates in transgenic mice overexpressing I-E α chains which inhibits the activation of B cells by some other indirect way. To explore this possibility, two types of chimeras were constructed, using nontransgenic or transgenic BXSB female mice lethally irradiated and reconstituted with BMC from transgenic and/or

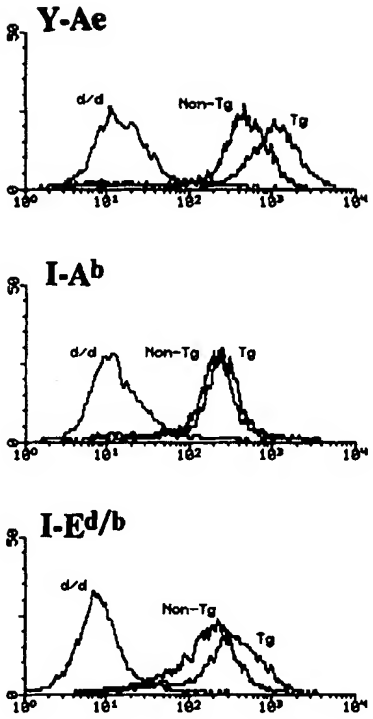

Figure 4. Increased surface expression of the Y-*Ae* epitope on surface IgM^+ B cells expressing the $E\alpha^d$ transgene in BXSB mice. Spleen cells from 2-mo-old transgenic and nontransgenic BXSB male mice of H-$2^{b/d}$ haplotype, obtained from a cross between the BXSB-E-1 and BXSB.H-2^d (7) mice, were first stained with FITC-conjugated anti-mouse μ chain mAb (LO-MM-9), and then incubated with biotinylated Y-*Ae*, Y-3P (anti-I-A^b), or Y-17 (anti-I-$E^{d/b}$) mAb, followed by PE-conjugated avidin. As controls, spleen cells from BXSB.H-2^d male mice were stained with Y-*Ae*, Y-3P, or Y-17 mAb.

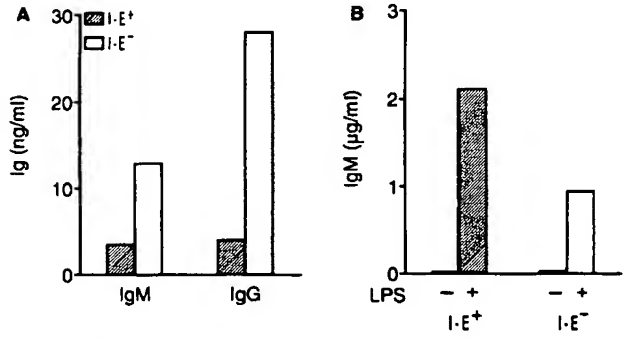

Figure 5. In vitro spontaneous IgM and IgG secretion (*A*), and LPS-stimulated IgM production (*B*) by $I\text{-}E^+IgM^+$ and $I\text{-}E^-IgM^+$ B cells isolated from the BXSB-E-1 mice. For the spontaneous IgM and IgG secretion, 10^5 $I\text{-}E^+IgM^+$ or $I\text{-}E^-IgM^+$ B cells sorted from spleen cells by a FACStar® were cultured for 24 h at 37°C in a humidified incubator containing 5% CO_2 in air. For the LPS-induced IgM production, 3×10^4 $I\text{-}E^+IgM^+$ or $I\text{-}E^-IgM^+$ B cells were stimulated with LPS for 5 d. IgM and IgG concentrations in supernatants were determined by ELISA. Representative results of three separate experiments are shown.

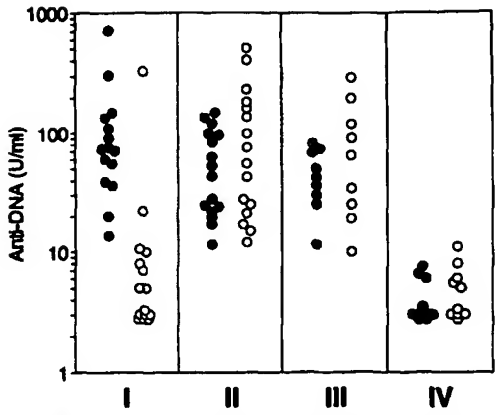

Figure 6. Serum levels of Igh^a (●) and Igh^b (○) anti-DNA Abs in four different groups of BMC chimeras (4-mo after reconstitution). Results are expressed in U/ml. The chimeras are those listed in Table 1.

nontransgenic BXSB male mice (Table 1). In these chimeras, the origin of B cells could be recognized because they bear different Igh allotypes. First, irradiated nontransgenic BXSB female mice were reconstituted with a mixture of BMC from nontransgenic (Igh^a) and transgenic (Igh^b) male BXSB mice (group I). These mice developed lethal glomerulonephritis by 8 mo after the reconstitution as did BMC chimeras not involving transgenic bone marrow, i.e., female BXSB mice reconstituted with nontransgenic male BMC (group III). Significantly, Igh allotype analysis of the anti-DNA Abs revealed that they all originated from the nontransgenic male bone marrow population (Fig. 6). Second, irradiated BXSB transgenic female mice were reconstituted with a mixture of Igh^a and Igh^b BMC of nontransgenic males (group II). These chimeras had a glomerular disease of the same severity, and comparable levels of anti-DNA Abs bearing in this condition the two Ig allotypes. It should be noted that chimeras reconstituted with a mixture of BMC from transgenic and nontransgenic female mice (group IV) failed to develop a comparable autoimmune syndrome.

All this shows that two explanations for the prevention of the autoimmune syndrome in the transgenic mice can be ruled out: I-E expression by cells of the transgenic animal or resulting from the graft of transgenic BMC did not lead to the generation of CD8$^+$ regulatory T cells "vetoing" the activation of autoreactive cells or to any form of clonal deletion of potential autoreactive T cells. It should be empha-

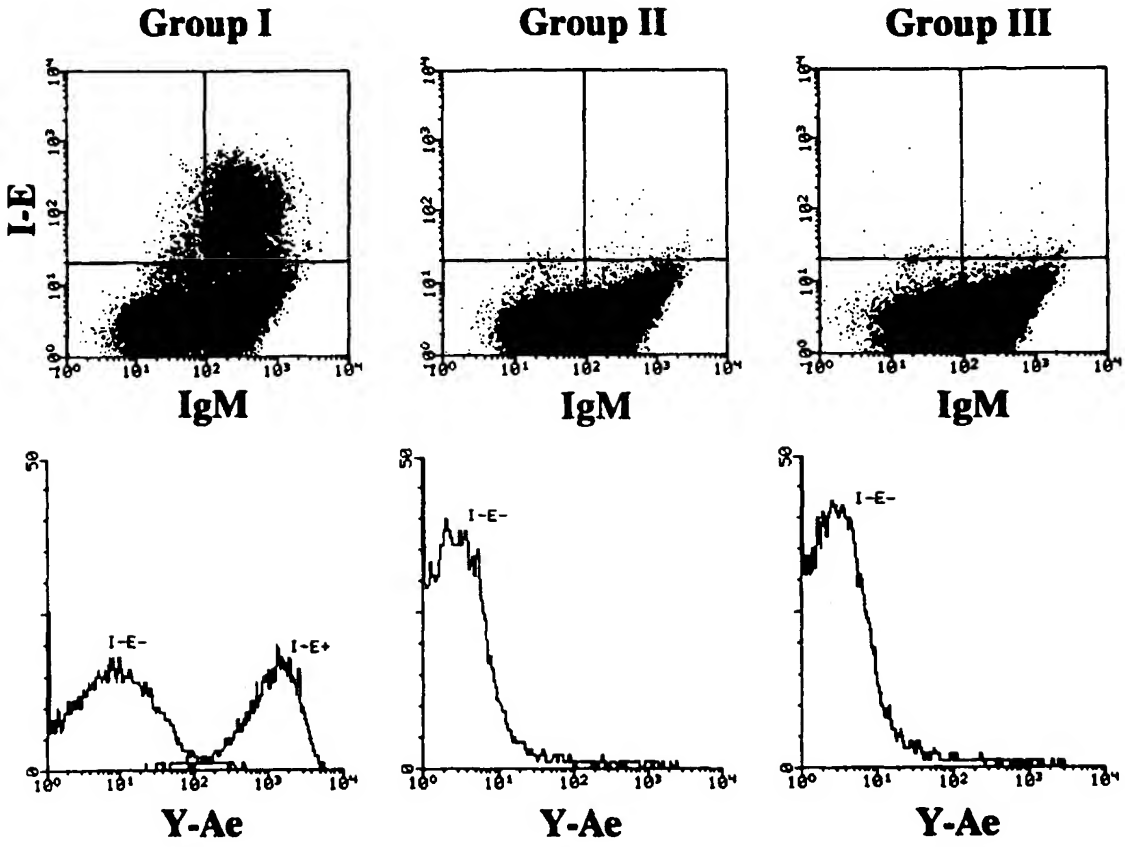

Figure 7. Surface expression of the Y-*Ae* epitope on I-E$^+$IgM$^+$ B cells from BMC chimeric mice. 2 mo after the reconstitution, spleen cells from three groups of BMC chimeras were first stained with FITC-labeled Y-17 (anti-I-E) mAb, then with PE-conjugated goat anti-mouse μ chain Abs, and finally with biotinylated Y-*Ae* mAb, followed by Streptavidin-RED670™. (*Top*) Surface I-E and IgM stainings in spleen cells. (*Bottom*) Expression of the Y-*Ae* epitope in the I-E$^+$IgM$^+$ and I-E$^-$IgM$^+$ B cells. Note the selective expression of the Y-*Ae* epitope in the I-E$^+$IgM$^+$ B cell population in mice of group I.

sized that the presentation of I-E α chain peptides by the I-A^b molecule, as determined by the Y-A*e* staining, was limited only to I-E^+ B cells derived from the transgenic bone marrow population in transgenic and nontransgenic double BMC chimeras (group I; Fig. 7), and that these B cells produced far less anti-DNA autoantibodies than I-E^- B cells in these chimeras (group I; Fig. 6). These results are entirely consistent with the hypothesis that a lower activation of B cells bearing an I-E α chain peptide in their I-A^b molecule is the mechanism preventing autoimmunity in the transgenic mice.

Our results suggest a novel mechanism explaining how the expression of a transgene encoding the I-E α chain prevents autoimmune diseases. Since I-E α chain–derived peptides apparently have a high affinity for I-A molecules other than the I-A^b (39), this mechanism might also be responsible for the protection against the development of autoimmune diabetes by the expression of an $E\alpha^d$ transgene in NOD mice (10–13). Further understanding of the protective mechanism(s) conferred by the $E\alpha^d$ transgene may elucidate the molecular and cellular bases central to the development of murine SLE and possibly of autoimmune diabetes. These mechanisms could have clinical implications in the design of future therapeutic strategies with self-peptides in several autoimmune disorders.

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Short paper

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The effects of a monoclonal antibody to interferon-γ on experimental autoimmune thyroiditis (EAT): prevention of disease and decrease of EAT-specific T cells

CBA/J mice immunized with thyroglobulin (Tg) develop an experimental autoimmune thyroiditis (EAT) with lymphocytic infiltration of the thyroid glands, autoantibodies to Tg and occurrence of EAT-specific T cells. When these mice were treated for 4 weeks after immunization with 1 mg/week of a monoclonal antibody (mAb) that neutralizes the activity of interferon-γ (IFN) a beneficial effect on the onset of EAT was observed. Characteristic features of EAT were significantly reduced, including the lymphocytic infiltrations of the thyroid glands and the serum levels of autoantibodies to Tg. Moreover, in lymphoid organs, mAb to IFN-γ significantly reduced the percentages of Tg-specific $CD8^+$ cells, labeled by the anti-clonotypic mAb AG7. These Tg-specific T cells seem responsible for thyroid damages and disease development, since EAT was simultaneously abrogated.
These results show that IFN-γ plays an essential role in the pathophysiology of EAT and suggest the possibility to treat autoimmune thyroid diseases with mAb to IFN-γ or drugs able to antagonize the production and/or the action of this cytokine.

1 Introduction

Hashimoto's thyroiditis (HT) and experimental autoimmune thyroiditis (EAT) are characterized by a marked lymphomonocytic infiltration of the thyroid gland, by hyperexpression of class I and *de novo* expression of class II MHC molecules on thyrocytes, and by the presence of circulating autoantibodies (A-Ab) to thyroid antigens (reviewed in [1, 2]).

IFN-γ, a cytokine produced by T lymphocytes and NK cells, may contribute to the pathogenesis of autoimmune thyroid diseases (ATD). Accordingly, studies on thyroid cells *in vivo* have shown that IFN-γ: (a) activates putative effectors of thyrocyte destruction such as T and B lymphocytes, macrophages and NK cells [1–2]; (b) enhances the immunogenecity of thyrocytes by up-regulating the expression of MHC class I and II antigens [3–6] and (c) impairs the growth of thyrocytes in combination with TNF-α [7]. The potential role of IFN-γ in the pathophysiology of ATD was further supported by our previous observation that EAT is induced in susceptible mice injected intrathyroidally with IFN-γ [8]. In addition, Frohman et al. [9] have shown that isologous thyroid specimens exposed *in vivo* to IFN-γ induce the T cell-dependent destruction of the thyroid lobes when implanted into adult isogeneic recipients; this effect depends upon the IFN-γ-induces expression of MHC class II antigens on thyrocytes [9].

Hence, we have studied the effects of the *in vivo* administration of one mAb that neutralizes murine IFN-γ on the development of EAT in CBA/J mice. We demonstrated that this mAb significantly reduced the percentages of $CD8^+$ thyroglobulin (Tg)-specific T cells responsible for thyroid damage in lymphoid organs. Simultaneously an abrogation of EAT was observed.

2 Materials and methods

2.1 Animals

Six- to eight-week-old CBA/J ($H\text{-}2^k$) mice, a strain susceptible to EAT [10, 11], were provided by Iffa Credo Breeding Center (Lyon, France). All animals were maintained under standard environmental conditions with free access to food and water. They were allowed to adapt during 1 week to their environment before initiating the experiment.

2.2 mAb

The rat mAb AN-18 is an IgG_{2a} anti-mouse IFN-γ that was produced and characterized by Prat et al. [12]. The antibody neutralizes the antiviral activity of both natural and recombinant mouse IFN-γ but does not react with murine IFN-α or IFN-β. In this experiment, mAb AN-18 was purified from hybridoma supernatants by passage onto an

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Abbreviations: **ATD:** Autoimmune thyroid diseases **EAT:** Experimental autoimmune thyroiditis **HT:** Hashimoto's thyroiditis **Tg:** Thyroglobulin **pTg:** Porcine thyroglobulin

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affinity column of sepharose beads coated with goat anti-rat Ig. The neutralizing titer of the An-18 mAb was 1×10^5 units mouse IFN-γ mg protein, as assessed, by the ability of AN-18 to suppress the capacity of IFN-γ to reduce the cytopathic effect produced by encephalomyocarditis virus (ATCC VR-129B) on L929 cells [13]. The L929 cell line (ATCC CCL 1) was maintained in RPMI 1640 medium supplemented with 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin. Control, isotype-matched, rat mAb was provided by Sera-Lab (Crawley Down, GB).

AG7 is an anti-clonotypic mAb (IgG_{2b}), specific for the TcR of cytotoxic T cells named HTC 2 and recognizing one pathogenic epitope of the Tg molceule in association with class I MHC antigens [14, 15]. It has been prepared [16] from splenocytes of CBA/J mice extensively immunized with HTC2 cells. AG7 blocks the capacity of HTC2 cells to induce lysis of target cells; it reacts with an HTC2 cell surface determinant which co-modulates with the CD3 antigen; it immunoprecipitates from ^{125}I-labeled solubilized HTC2 cell membrane products of 90 and 72 kDa under nonreducing conditions and 6 kDa under reducing conditions. We demonstrated that 40 μg of this reagent could prevent the onset of EAT when injected 1 day prior to immunization [16].

2.3 Induction of EAT

Pig Tg (pTg) from Sigma (St. Louis, MO) was emulsified in CFA for immunization on day 0 and in IFA for challenge on day 14. CFA suspension contained 1 mg/ml of *Mycobacterium tuberculosis*, strain H37 Ra (Difco Lab, Detroit, MI). Each injection was performed s.c. with 100 μg of pTg. Animals were killed on day 28 post immunization.

2.4 Histopathological studies of thyroid specimens

The histological severity of EAT was assessed by blind evaluation of thyroid specimens obtained at the end of the study (day 28 post immunization). Infiltration indexes were evaluated on 5-μm-thick sections stained with Masson Goldner's trichrome solution. The histological incidence of EAT was graded as function of the mononuclear cell thyroid infiltration as described elsewhere [17]: (1) interstitial accumulation of inflammatory cells distributed between two or more follicles; (2) one or two foci of inflammatory cells reaching at least the size of one follicle; (3) 10% to 40% of the thyroid replaced by inflammatory cells; and (4) > 40% of the thyroid replaced by inflammatory cells. Mean grades of EAT were assigned as follows: 0 to 1 negative, 1 to 2 mild, 2 to 3 severe, and 3 to 4 acute. The group of mice with the highest lymphocytic infiltration index value were considered to exhibit 100% EAT incidence (group D, see Table 1). The percentages of EAT incidence in the two groups of mice treated with the low and high dose of mAb AN-18 were calculated by subtracting the infiltration index (II) of nonimmunized mice (0.8%, 0% EAT incidende) from the values of each group, including the control group, and the results calculated as follows: EAT incidence group A = (II group A-II group C)/(II group D-II group C) × 100; EAT incidence group B = (II group B-II group C)/(II group D-II group C) × 100.

2.5 Anti-Tg antibodies

Anti-Tg A-Ab were assayed by a solid-phase ELISA as described in detail elsewhere [17]. In brief, flat-bottom microtiter plates (Costar, No. 3590, Cambridge, MA) were coated overnight at 4 °C with 100 μl of 100 μg/ml native pTg. After washing with PBS-Tween 20 (PBS-T), the free protein binding sites were blocked by adding 1.5% BSA for 2 h at 37 °C. Sera from individual mice, diluted (PBS-T) 1/100, 1/1000 and 1/10000, were incubated for 2 h at 37 °C and washed out extensively. Alkaline phosphatase-conjugated goat anti-mouse IgG (Miles-Yeda Laboratories, Rehovot, Israel), diluted (PBS-T) 1/500, was added as second antibody and the colorimetric reaction revealed by substrate addition. The plates were read with a Titertek multiscan spectrophotometer (Flow Labs, Rockville, MD) at 405 nm.

2.6 Fluorescence analysis of AG7$^+$ T cells and statistical analysis

Spleen and lymph node cell samples were collected, adjusted at 1×10^6/ml and incubated (30 min at 4 °C) with 100 μl of 5 μg/ml FITC-conjugated AG7 mAb. Double staining was performed using FITC-conjugated AG7 and PE-coupled anti-murine CD8 mAb. Appropriate FITC-coupled mAb E1 was used as control in each analysis. After washes with PBS containing 0.1% NaN_3 and 5% FCS, the cells were washed and fixed with 1% formaldehyde. Analysis was performed using a cytofluorograph FACScan (Becton Dickinson, Mountain View, CA). In each aliquot, 10000 cells were counted. Percentages were noted and values expressed as percentages of labeled cells ± SEM. This methodology discriminates cell subsets as little as 0.1% of a cell suspension using two different surface markers [18]. Cells were defined as follows: AG7$^+$ cells = (FITC-AG7$^+$ cells) − (FITC-E1$^+$ cells). AG7$^+$CD8$^+$ cells = (FITC-Ag7$^+$PE-CD8$^+$ cells) − (FITC-E1$^+$ PE-CD8$^+$ cells).

Statistical analysis was performed by ANOVA test.

3 Results

EAT, induced in CBA/J mice as described in Sect. 2.3, reached an acute phase after 28 days, as reflected by the severe mononuclear cell infiltration of the thyroid glands and the increased blood titers of anti-Tg A-Ab. To interfere with the initial steps of the disease some animals were treated with AN-18, a rat mAb to mouse IFN-γ, or with a control rat mAb, twice a week for 4 weeks after immunization.

Mice immunized with pTg and receiving 1 mg/week of control mAb (Table 1, group D) showed lymphoid infiltration of the thyroid glands and increased serum titers of anti-Tg A-Ab similar to untreated CBA/J mice that were immunized with pTg (data not shown). In contrast, animals immunized with pTg and injected with 1 mg/week of AN-18 mAb (Table 1; group B) showed a substantial reduction (52%) of the lymphocytic infiltration of the thyroid glands and a slight, although significant, decrement of serum levels of autoantibodies to Tg (Table 1). Mice treated with

Table 1. Effects of administration of mAb to murine IFN-γ (AN-18) on EAT[a]

Group of mice (number)	Immunization[b]	mAb (μg)[c]	Lymphocytic infiltration indexes	Incidence of EAT	A-Abs to pTg[d] (Absorbance)
A (14)	pTg	200	2.3 ± 0.6*	60% (40)[e]	607 ± 47***
B (19)	pTg	1000	2.0 ± 0.4**	48% (52)[e]	574 ± 38****
C (10)	Adjuvants only	0	0.8 ± 0.4	0%	17 ± 6
D (9)	pTg	1000[f]	3.3 ± 0.5	100%	738 ± 33

a) Statistical analysis was performed by ANOVA:
* not significant (NS) *vs.* group D;
** $p < 0.02$ *vs.* group D;
*** NS *vs.* group D;
**** $p < 0.05$ *vs.* group D.

b) pTg (100 μg s.c.) in CFA on day 0, in IFA on day 14.
c) Twice a week, 100 and 500 μg i.p., during the 4 weeks of the experiment.
d) Sera diluted 1:10000.
e) () = % of EAT protection.
f) Isotype-matched control mAb.

Table 2. Effects of mAb AN-18 on percentages of AG7$^+$ or Ag7$^+$CD8$^+$ T cells in lymphoid organs from pTg-immunized mice. (Day 28 post immunization; mean ± SEM)[a]

Group of mice	Spleen cells		Lymph node cells	
	Ag7$^+$	AG7$^+$CD8$^+$	AG7$^+$	AG7$^+$CD8$^+$
A	1.93 ± 0.42**	0.99 ± 0.35*	0.64 ± 0.22*	0.25 ± 0.09
B	1.96 ± 0.23**	1.05 ± 0.17*	1.16 ± 0.28*	0.59 ± 0.23*
C	1.31 ± 0.20	0.96 ± 0.12	0.83 ± 0.24	0.52 ± 0.13
D	3.86 ± 1.23***	2.42 ± 0.76***	2.50 ± 0.45***	1.11 ± 0.53*

a) Each group was compared to group C: * NS, ** $p = 0.05$, *** $p < 0.001$.

0.2 mg/week of AN-18 (Table 1, Group A) still exhibited a 40% reduction of the lymphocytic infiltration without a significant reduction of anti-Tg A-Ab.

Knowing that EAT is a T cell-mediated disease [1], we wondered whether AN-18 could develop its inhibitory effect by acting at the T cell level and, more precisely, at the level of T cells responsible for thyroid damage, *i.e.* Tg-specific cytotoxic T cells. To prove this, we have used the AG7 mAb. This anti-clonotypic mAb recognizes TcR of T cells that induce lysis of syngeneic thyroid epithelial cells in a class I-restricted context, or of syngeneic macrophages pulsed with either Tg or one Tg epitope inducing EAT [15]. We have investigated whether AN-18 administration prevented the occurrence of Tg-specific AG7$^+$ and AG7$^+$CD8$^+$ T cells.

Mice injected with adjuvants alone showed the lowest percentages of AG7$^+$ or CD8$^+$AG7$^+$ T lymphocytes in their spleens and lymph nodes (Table 2, group C); in contrast, the number of these cells significantly increased in the lymphoid organs of pTg-immunized mice receiving the control mAb which develop severe EAT (Table 2, group D). This increase was not observed in mice treated with 1 or 0.2 mg/week of mAb AN-18, which behaved as controls (Table 2, groups A and B).

4 Discussion

IFN-γ is a lymphokine exhibiting pleiotropic effects on the immune response (see [19] for a review). The maturation of cytotoxic and helper T lymphocytes requires both IFN-γ and IL-2. IFN-γ enhances IgG_{2a} synthesis, whereas it completely inhibits synthesis of other IgG subclasses and of IgE. Antigen presentation may be enhanced by IFN-γ through the up-regulation of the expression of MHC class II antigens. Finally, macrophages are activated by IFN-γ to secrete proinflammatory and cytotoxic molecules including IL-1 and TNF-α.

The possible contribution of IFN-γ to the development of autoimmune disorders has recently been a matter of interest. The use of IFN-γ or IFN-γ antagonists in animal models may be useful to highlight pathogenic mechanisms and to evaluate novel therapies for the treatment of human autoimmune diseases. Along this line of research, we and others have shown that the *in vivo* treatment with mAb to IFN-γ exerted beneficial effects on the development or the course of type-1 diabetes [20–22], systemic lupus erythematosus [23], and chronic endogenous uveitis [24] in experimental models; in contrast, anti-IFN-γ mAb exacerbated the symptoms of experimental allergic encephalomyelitis [25] and heterogeneously modulated the course of adjuvant-induced arthritis [26–27].

In this study, the *in vivo* treatment with one mAb that neutralizes endogenous IFN-γ (AN-18) significantly reduced the development of EAT in CBA/J mice. This finding is consistent with the observed induction of EAT in the same strain of mice injected intrathyroidally with IFN-γ [8] and strongly suggests a pathogenic role for IFN-γ in the development of EAT.

The mechanisms by which AN-18 prevents EAT are apparent in the present study. The anti-IFN-γ mAb mainly

acts by diminishing the percentages of Tg-specific cytotoxic $AG7^+CD8^+$ T cells. Moreover, the slight decreases in serum titers of anti-Tg A-AB observed in the AN-18-treated mice suggest that the mAb has also modulated the function of autoreactive helper T and B lymphocytes. However, the interaction with other putative pathogenic mechanisms cannot be excluded. For example, AN-18 may induce a down-regulation of the expression of MHC class II molecules on thyrocytes (which is induced *in vitro* by IFN-γ [4, 7]), and thus may explain [28] the reduced lymphoid infiltration of the thyroid glands. Through this mechanism AN-18 may prevent thyrocytes from acquiring an antigen-presenting cell function [29], therefore providing another potential mechanism by which the autoimmune reaction could have been hampered.

In conclusion, these data show for the first time that blockade of endogeneous IFN-γ with a specific mAb effectively prevents the onset of EAT. Moreover, this study provides data on the effect of mAb to IFN-γ on T cells responsible for thyroid gland damage. These findings warrant studies to evaluate the possibility to treat HT patients with antibodies to IFN-γ or with other IFN-γ antagonists.

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